2002

Primaquine-Induced Hemolytic Anemia: Formation and Mechanism of Action of the Hemotoxic Metabolite 6-Methoxy-8-Hudroxylamoniquinoline (MAQ-NOH)

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Primaquine-Induced Hemolytic Anemia: Formation and Mechanism of Action of the Hemotoxic Metabolite 6-Methoxy-8-Hydroxylamoniquinoline (MAQ-NOH)

by

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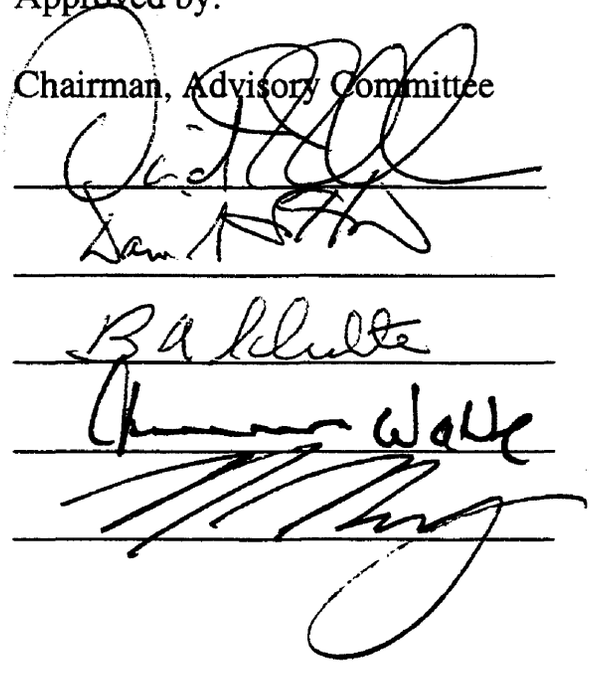
A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies

Department of Cell and Molecular Pharmacology and Experimental Therapeutics

2002

Approved by:

Chairman, Advisory Committee



To my husband Brian and children Joseph and Emma

Acknowledgements

I wish to express my utmost gratitude to my mentor, Dr. David McMillan, for his patience and understanding during my graduate training. Through your insistence that I make my own decisions, and providing such a stimulating scientific environment, you have nurtured my ability to think both as a scientist and as an individual.

I would also like to thank Dr. David Jollow for his continuous creative stimulation and endless hours of insightful discussions. These were an integral part of my growth as a scientist.

I also thank my committee members for their genuine interest, support and guidance throughout my training:

Dr. Thomas Walle
Dr. Brad Schulte
Dr. Robert Means

Special thanks to the past and present members of the "Jollow/McMillan lab" including Zinat Hassanpour, Dr. JoEllyn McMillan, and Dr. Jennie Walgren for their support, advice, and friendship over the past five years. In particular I would like to thank Jennifer Schulte for her amazing patients in teaching me all the techniques applied in this dissertation. You have been a great source of support and encouragement both as a coworker and a friend.

Thanks to the following individuals for their significant contribution in various aspects of this project:

Dr. Kevin Schey and the Mass Spectrometry facility for his help in identifying the 6-MAQ microsomal metabolite.
Dr. Andrew Gelasco and the EPR facility for his help in both generating and interpreting the MAQ-NOH-induced free radical data.
Dr. John Oatis and the NMR facility for his help in confirming the purity of the synthetically produced MAQ-NOH.
Nancy Smythe and the SEM facility for her help in the preparation of the MAQ-NOH-treated red blood cells for morphology studies.

I want to especially thank Alema Galijatovic and Lauren Ball. Alema, your support and friendship, even over the long distances, helped me to believe in myself and kept me focused. And Lauren, thank you for all your friendship and advice, for being there in all the good times, and for being a shoulder for me to lean on when I needed one. The office will never be quite the same!

I thank my parents for instilling in me the desire to pursue my doctorate and for providing the love and support I needed to succeed.

And to my husband, I thank you for believing in me. I never could have achieved this lifelong dream without you.

Copyright Information

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Key to Abbreviations

6-MAQ	6-methoxy-8-aminoquinoline
5-OH-PQ	5-hydroxyprimaquine
5,6-DHAQ	5,6-dihydroxy-8-aminoquinoline
5,6-DHPQ	5,6-dihydroxyprimaquine
^{51}Cr	Chromium-51
DDS-NOH	dapsone hydroxylamine
DEM	diethyl maleate
DMSO	dimethyl sulfoxide
EMPO	2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide
EPR	electron paramagnetic resonance
ESI	electrospray ionization
G6PD	glucose-6-phosphate dehydrogenase
GS-protein	glutathione-protein mixed disulfides
MS	mass spectrometry
MAQ-NOH	6-methoxy-8-hydroxylaminoquinoline
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PQ	primaquine
PQ-CX	carboxyprimaquine
PS	phosphatidyl serine

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Abstract

Primaquine is an important antimalarial drug that is often dose-limited in therapy by the onset of hemolytic anemia. It is well accepted that this toxicity is due to the action of its metabolites and not the parent compound. However, the toxic species have not been identified and little is known about the mechanism underlying primaquine-induced red cell injury. Previous studies to identify the hemotoxic metabolites have focused on the redox active phenolic derivatives of primaquine and have shown that a number of these compounds are capable of decreasing red cell GSH levels and oxidizing oxyhemoglobin to methemoglobin. However, these derivatives have not been detected in humans following primaquine administration. Since N-hydroxy metabolites are known to mediate the hemotoxicity of several other arylamines, this dissertation addresses the possibility that an N-hydroxy metabolite of primaquine may play a role in the hemotoxicity of the parent compound.

Metabolic studies in rat and human microsomes determined that the known human primaquine metabolite, 6-methoxy-8-aminoquinoline (6-MAQ) is N-hydroxylated to 6-methoxy-8-hydroxylaminoquinoline (MAQ-NOH). MAQ-NOH was shown to be hemolytic *in vivo* in rats, and directly hemotoxic in rat erythrocytes. These observations suggest that primaquine can be metabolized to MAQ-NOH and that this metabolite has the requisite properties to play a role in the hemotoxicity of the parent compound.

In regard to the mechanism underlying MAQ-NOH hemolytic activity, it was demonstrated that under hemolytic conditions, MAQ-NOH induced an oxidative stress within red cells, but the pattern of responses was quite different than other N-hydroxylamines previously shown to have direct hemolytic activity.

In this dissertation, data is presented to suggest that MAQ-NOH may induce hemolytic injury in the red cell by more than one mechanism; one that involves lipid peroxidation in the presence of normal amounts of erythrocytic GSH, and one that involves protein oxidation in red cells with low levels of GSH, as seen in individuals with glucose-6-phosphate dehydrogenase deficiency. The relative contribution of MAQ-NOH versus the phenolic type derivatives in mediating this toxicity remains to be assessed.

CHAPTER 1

Introduction

Drug-Induced Hemolytic Anemia

Hemolytic anemia occurs when red blood cells are removed prematurely from the circulation and the bone marrow is unable to compensate for the loss. It has been a well-recognized toxic side effect of drugs for many years with the first case reports on the capacity of phenacetin to induce anemia documented in the 1890's (Cleaves, 1892; West, 1893). While initially the problem of drug-induced hemolytic anemia was given little attention, it became of great social and military concern during World War II and the Korean War in response to a high incidence of hemolytic anemia in otherwise healthy military personnel prophylactically administered primaquine for malaria (Beutler, 1959).

In addition to primaquine, many other compounds are known to induce a hemolytic response (Beutler, 1959). These include a wide range of structurally and functionally diverse compounds (Table 1.1) such as the arylamine drug dapsone, the antibacterial agent chloramphenicol, and the herbicide propanil. While there does not appear to be any link in their pharmacological activity that predisposes these compounds to be hemolytic agents, many of them contain an arylamine nucleus (fig 1.1) or can be metabolically converted to an aromatic amine *in vivo*.

Glucose-6-Phosphate Dehydrogenase Deficiency

It was early recognized that hemolytic anemia in response to primaquine was most pronounced in a particular sub-population of individuals. These include 10-15% of African Americans (A-) and some Caucasians of Mediterranean descent (Med-), the

Table 1.1
Compounds Known to Induce Hemolytic Anemia

Analgesics
- Acetanilid
- Acetylsalicylic acid (*)
- Antipyrine
- Phenacetin (*)
- Pyramidone

Sulfonamides and Sulfones
- Sulfanilamide
- Sulfapyradine
- Dapsone
- Sulfacetamide
- Sulfisoxazole (Gantrisin) (*)
- Thiazolsulfone
- Sulfoxone (*)

Antimalarials
- Primaquine
- Pamaquine
- Pentaquine
- Quinacrine (Atabrine)

Nonsulfonamide Antibacterial Agents
- Furazolidone
- Nitrofurantoin
- Nitrofurazone
- Chloramphenicol (***)
- Paraaminosalicylic acid

Miscellaneous
- Napthalene
- Trinitrotoluene
- Methylene Blue (*)
- Nalidixic acid
- Dimercaprol(*)
- Phenylhydrazine
- Quinine (**)
- Quinidine(**)

(*) Slightly hemolytic in African Americans, or only in very large doses
(**) Hemolytic in Caucasians, but not in African Americans
(***) Possibly hemolytic in Caucasians, but not in African Americans or Orientals

(from Beutler, 1972)

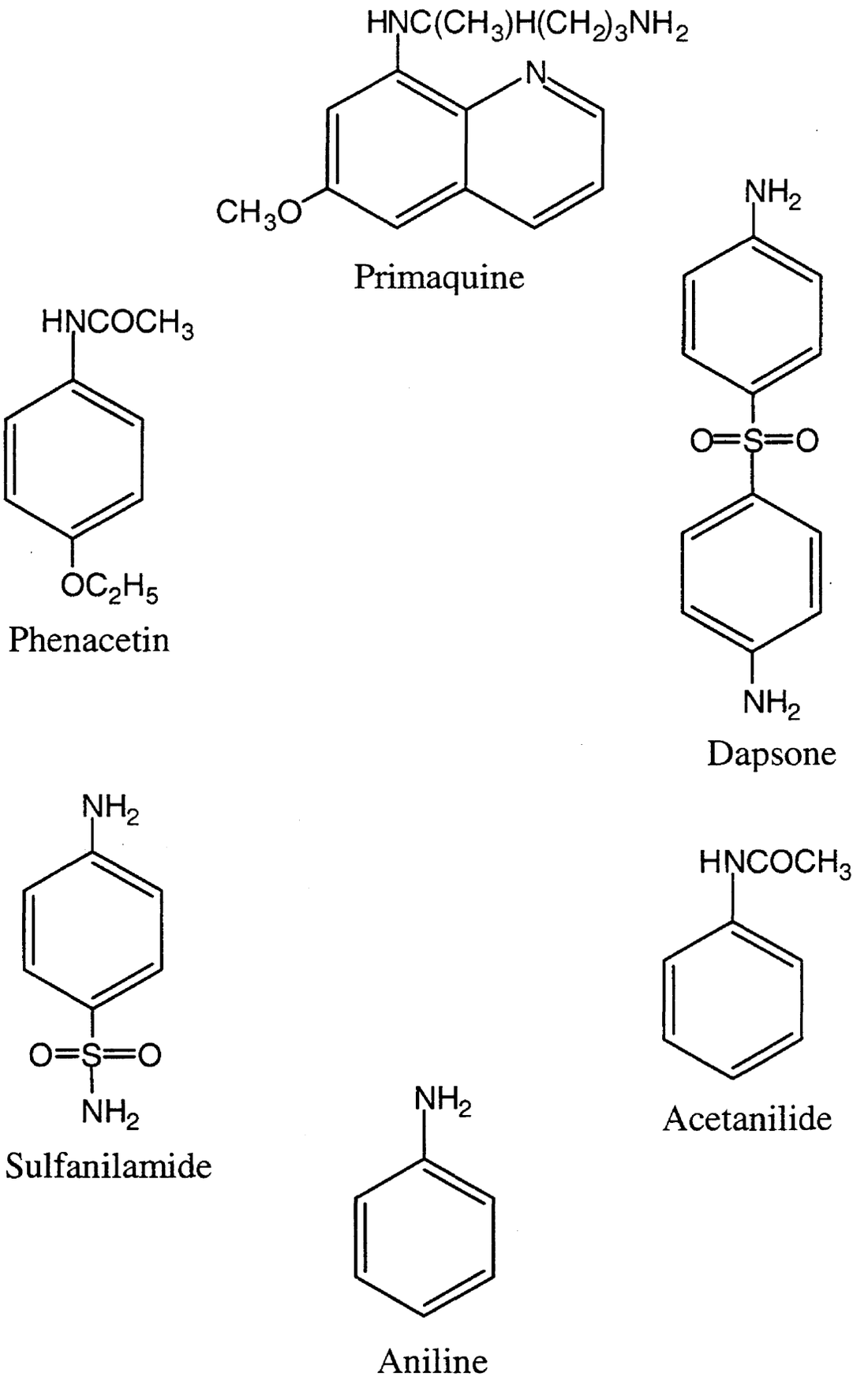

Fig. 1.1. Structural formulas of various drugs known to induce hemolytic anemia in humans

percentage depending on geographical location (Beutler, 1972). This sensitivity was later associated with an intrinsic genetic defect in the enzyme glucose-6-phophate dehydrogenase (G6PD). G6PD deficiency is an X-linked recessive trait that affects up to 400 million people worldwide (for review see Beutler, 1990). While it is recognized that multiple variants of this enzyme deficiency exist, a majority of them result from a single point mutation that causes an amino acid change in the protein. It is thought that these mutations occur within the dimer interface of the protein, destabilizing the dimer, and exposing the monomeric forms to enhanced degradation (Mason, 1996). Thus, the mutated enzyme has a normal level of activity but a shorter cellular lifespan. Due to the lack of protein turnover in red cells, G6PD activity is lost as the erythrocyte ages. Erythrocytes are considered depleted of the enzyme activity after 55 days and 20 days in A- and Mediterranean variants, respectively.

G6PD is the first enzyme in the hexose monophosphate shunt metabolic pathway and is solely responsible for generating NADPH in erythrocytes. NADPH is an essential cofactor for the antioxidant enzyme catalase and the glutathione peroxidase/ reductase enzyme system (Kirkman and Gaetani, 1984). Therefore, while G6PD-deficient individuals may not experience any pathology under normal conditions, they are unable to maintain adequate levels of NADPH and therefore GSH when challenged with an oxidative stress.

Malaria

The Disease

In recent literature, malaria was recognized as one of the world's most devastating human infectious diseases responsible for an estimated 1.5-2.7 million deaths each year, most of which are children under the age of five (Phillips and Phillips-Howard, 1996; Miller, 1997). Furthermore, another 300-500 million people are reported to be infected with the parasite each year and an additional 2.4 billion people are potentially exposed to the parasite on a daily basis (Phillips and Phillips-Howard, 1996; Miller, 1997). Although a majority of malaria cases occur in sub-Saharan Africa, it is believed that more than 100 countries throughout Southeast Asia, the Indian subcontinent, the South Pacific region and Latin America are burdened with this problem (Kondrachine and Trigg, 1997). In addition, over 1000 cases of malaria per year have been reported in the last decade to the US Centers for Disease Control; most of these are due to international travelers returning from endemic areas (Kondrachine and Trigg, 1997; Magill, 1998).

Parasite Life Cycle

Human malarial infections are caused by four species of the plasmodial parasite, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Tracy and Webster, 1996). Individuals primarily contract malaria following a bite from an infected female *Anopheles* mosquito. While feeding, the mosquito releases sporozoites from their salivary glands into the bloodstream of an individual. Once in the bloodstream, the sporozoites are quickly taken up by the liver and begin the primary tissue phase of infection. During this

phase, the sporozoites multiply and develop within hepatocytes into mature tissue schizonts. After 5-16 days, depending on the species, the schizonts rupture and release thousands of merozoites into the circulation where they invade red blood cells, initiating the erythrocytic or blood stage of malaria. Of importance, upon release from the liver, the parasite is unable to re-infect other tissues. However, in *P. vivax* and *P. ovale* infections, some of the parasites remain in the liver, giving rise to the latent tissue form of the parasite. This form of the parasite is released into the bloodstream at a later date, thus causing relapses. Once in the red cell, most of the plasmodia continue to multiply and develop into schizonts until the red cell ruptures, again releasing merozoites that can further infect additional erythrocytes. However, some of the parasites will differentiate sexually within the red cells into gametocytes. It is this form of the parasite that is ingested by an uninfected mosquito and ultimately develops within the mosquito into a sporozoite that can infect another individual following the infected mosquito's next blood meal.

Therapy

The antimalarial drugs in use today are active against different stages of the malaria parasite (Tracy and Webster, 1996). However, a majority of them are used clinically to target either the erythrocytic or the tissue forms of the species. For example, both chloroquine and quinine are known to have gametocidal activity but are primarily used to kill the asexual blood forms of the parasite. Additional agents active against the blood forms of malaria include mefloquine and halofantrine. Primaquine is an antimalarial drug of particular value in that it has been shown to be active against multiple stages of the

parasite. These include the erythrocytic stages of *P. vivax*, the primary hepatic forms of *P. falciparum*, and the gametocidal form of all four malaria species (Grewal, 1981; Peters, 1999). In addition, primaquine is known to be highly effective in treating the persistent exoerythrocytic, or tissue, form of malaria and therefore is particularly important clinically for the radical cure of *P. vivax* and *P. ovale* infections.

Drug Resistance

The widespread emergence of drug resistant parasites has been the subject of several recent reviews (Kondrachine and Trigg, 1997; Foley and Tilley, 1998; Milhous and Kyle, 1998). The alarming resurgence seen in malaria cases is thought to be largely due to this problem. For example, chloroquine resistance has been documented in almost all malaria endemic areas except Central America (Kondrachine and Trigg, 1997). As this was once the drug of choice for malaria, its clinical effectiveness has been drastically reduced in the last 20 years. Furthermore, resistance to the alternative sulfadoxine-pyrimethamine is now rampant in Africa, Southeast Asia and South America (Kondrachine and Trigg, 1997; Milhous and Kyle, 1998). As an alternative course of treatment, infected individuals are being administered the more expensive antimalarial agents such as mefloquine, quinine and halofantrine, however, an alarming number of cases of resistance to these agents are being reported as well (Foley and Tilley, 1998; Milhous and Kyle, 1998).

Primaquine

In response to a shortage of the antimalarial agent quinine during World War II, the US initiated a large research program to develop more effective, safer antimalarial drugs (Greenwood, 1995). From this program, emerged the 8-aminoquinoline, primaquine, synthesized by Robert Elderfield (Elderfield et al., 1955). Extensive use in American soldiers suffering from relapsing *vivax* malaria during the Korean War (Garrison et al., 1952; Alving et al., 1953) helped to establish the clinical value of primaquine as the drug of choice for the radical cure of malaria.

Primaquine continues to be the only drug used clinically today to eliminate the latent tissue forms of *P. vivax* and *P. ovale* infections. Moreover, recently the gametocytocidal activity of primaquine has been exploited in its co-administration with blood schizontocidal drugs like chloroquine in an effort to combat the widespread drug resistance to these agents particularly in *P. falciparum* infections, the most deadly species of malaria in humans. (Baird et al., 1995; Peters, 1999). Of interest, the clinical use of primaquine has also recently expanded to include treatment of *Pneumocystis carinii* pneumonia in patients with acquired immunodeficiency syndrome (Black et al., 1991; Toma, 1991; Kantor, 1992).

Mode of Action

Despite its use for more than forty-five years, the therapeutic mode of action of primaquine is unknown. In particular, it is not understood why primaquine is more effective against the hepatic stage, than it is against the asexual blood stages of the

parasite. Furthermore, the question remains as to whether primaquine antimalarial activity is due to the parent compound or its metabolites. One problem facing investigators in trying to elucidate this mechanism is the lack of a well-developed *in vitro* culture system for the latent tissue stages of *P. vivax* and *P. ovale*.

While attempts to develop an *in vitro* culture systems for the human malaria species have not been successful, two rodent forms, *P. yoelii* and *P. berghei*, have been cultured *in vitro*. In this regard, Bates *et. al.* (Bates et al., 1990) synthesized a variety of primaquine analogs and in comparison studies using *P. berghei* infected HepG2 cells determined that certain primaquine derivatives were much more potent antimalarial agents than primaquine. These investigators further demonstrated that these derivatives displayed a linear correlation between spontaneous superoxide formation and antimalarial activity suggesting that primaquine is converted in the liver to redox active intermediates that contribute to the parent compound antimalarial activity by inducing an oxidative stress within the parasite. These data are consistent with previous studies in which redox active compounds like alloxan, phenylhydrazine, and divicine were shown to suppress parasitaemia in malaria-infected mice (Clark and Hunt, 1983; Clark et al., 1984b; Clark et al., 1984c).

In contrast, a second group of investigators have proposed that primaquine itself exerts its antimalarial activity by binding the *Plasmodium* ubiquinone binding site on cytochrome b, thereby interrupting the parasite's mitochondrial respiratory chain (Vaidya et al., 1993). Specificity has been attributed to the unique structural features of the parasite ubiquinone-binding site. In support, when tested *in vivo* in rats infected with *P. yoelii*, primaquine induced thickening of the parasite mitochondrial membranes as well as

dramatic mitochondrial swelling (Boulard et al., 1983; Bates et al., 1990) with no effect on host hepatocyte mitochondria. Although not a hypnozonticidal antimalarial agent, atovaquinone is known to mediate its activity via this mechanism (Fry and Pudney, 1992; Srivastava et al., 1997).

Resistance

There have recently been some reports in the literature of *P. vivax* resistance to primaquine treatment in Papua New Guinea, Southeast Asia, and Central and South America (Krotoski, 1980; Arias and Corredor, 1989; Luzzi et al., 1992; Bunnag et al., 1994; Gascon et al., 1994; Nayar et al., 1997). In support of these observations, resistance has been induced experimentally to the blood schizontocidal activity of primaquine in a number of malaria species including *P. vivax* in humans (Arnold et al., 1961; Thompson, 1967). However, as pointed out by Collins *et. al.,* (Collins and Jeffery, 1996) these cases are often in endemic areas that are infested with particularly virulent strains of *P. vivax* (i.e., Chesson strain from New Guinea) that are known to require higher doses of primaquine to be eradicated, which suggest that these are cases of tolerance not resistance. In addition, subsequent examination of many of these reports has determined that sub-therapeutic dosing, lack of compliance, and re-infection may also contribute to what appeared to be relapses (Collins and Jeffery, 1996; Looareesuwan et al., 1997).

As a result of the widespread parasite resistance to many of the blood schizontocides and possibly to primaquine, it is imperative that new antimalarial agents be developed. To do so, researchers need to understand the mechanism of action, toxicity, and parasite

resistance to the existing drugs in order to rationally design more effective drugs with a higher therapeutic index.

Primaquine Hemotoxicity

Primaquine therapeutic and prophylactic use is limited by its toxic side effects, methemoglobinemia and hemolytic anemia (Tarlov et al., 1962; Carson et al., 1981; Clyde, 1981). Early clinical studies in G6PD A- deficient individuals characterized the course of the hemolytic response into three phases: acute, recovery, and resistant phases (Dern et al., 1954). During the acute hemolytic phase, up to 50% of the red cells may be lost from the circulation. A decrease in blood GSH levels, a transient increase in methemoglobin levels and the appearance of intraerythrocytic inclusions (Heinz bodies) have been reported to accompany this phase.

Despite continuing primaquine therapy, these patients begin to recover in 7-12 days following administration of the drug. Beutler and others (Kellermeyer et al., 1961; Beutler, 1971) have shown that this self-limiting hemolytic response in A- individuals is due to the selective destruction of older erythrocytes with decreased G6PD enzyme activity. As the red cell population becomes enriched in younger cells, the patient becomes "resistant" to the hemolytic response. Although the surviving cells become sensitive to primaquine as they age and are removed from circulation, their loss is compensated for by erythropoiesis. If drug treatment is discontinued, however, full susceptibility returns within 4-6 months as the red cells return to the normal pre-drug age distribution.

In contrast, the hemolytic response in the Mediterranean variant of G6PD deficiency is not self-limiting (George et al., 1967). In Med- individuals the G6PD activity is markedly decreased in both young and old cells (Piomelli et al., 1968). Thus a hemolytic response in these individuals may be much more severe than in an A- variant, perhaps even life-threatening.

Primaquine Metabolism

It is interesting that despite the importance of metabolism in regard to the mode of action and toxicity of primaquine (Tarlov et al., 1962; Fraser and Vesell, 1968; Fletcher et al., 1988; Bates et al., 1990), its metabolic fate remains poorly defined. The chemical instability of the putative primaquine metabolites, along with their poor recovery from biological systems has significantly contributed to the difficulty of this problem (Strother et al., 1984; Idowu et al., 1995). Pharmacokinetic studies of primaquine in humans have determined that drug bioavailability is 96%, half-life is 6 hours, and volume of distribution is 250 L indicating extensive uptake into the tissues (Mihaly et al., 1984; Mihaly et al., 1985). Therefore since primaquine is taken chronically (or sub-chronically) for malaria, the potential exists for drug and/or metabolite accumulation *in vivo*. Furthermore, less than 2% of the administered dose of primaquine is renally excreted (Mihaly et al., 1984).

The primary metabolic pathways of primaquine (fig. 1.2) are thought to involve oxidative metabolism of both the aminoquinoline ring and the alkylamino side-chain of the molecule (Strother et al., 1981; Price and Fletcher, 1986; Idowu et al., 1995). Primaquine-3'-carboxylic acid (PQ-CX) has been found in the plasma of humans (Mihaly

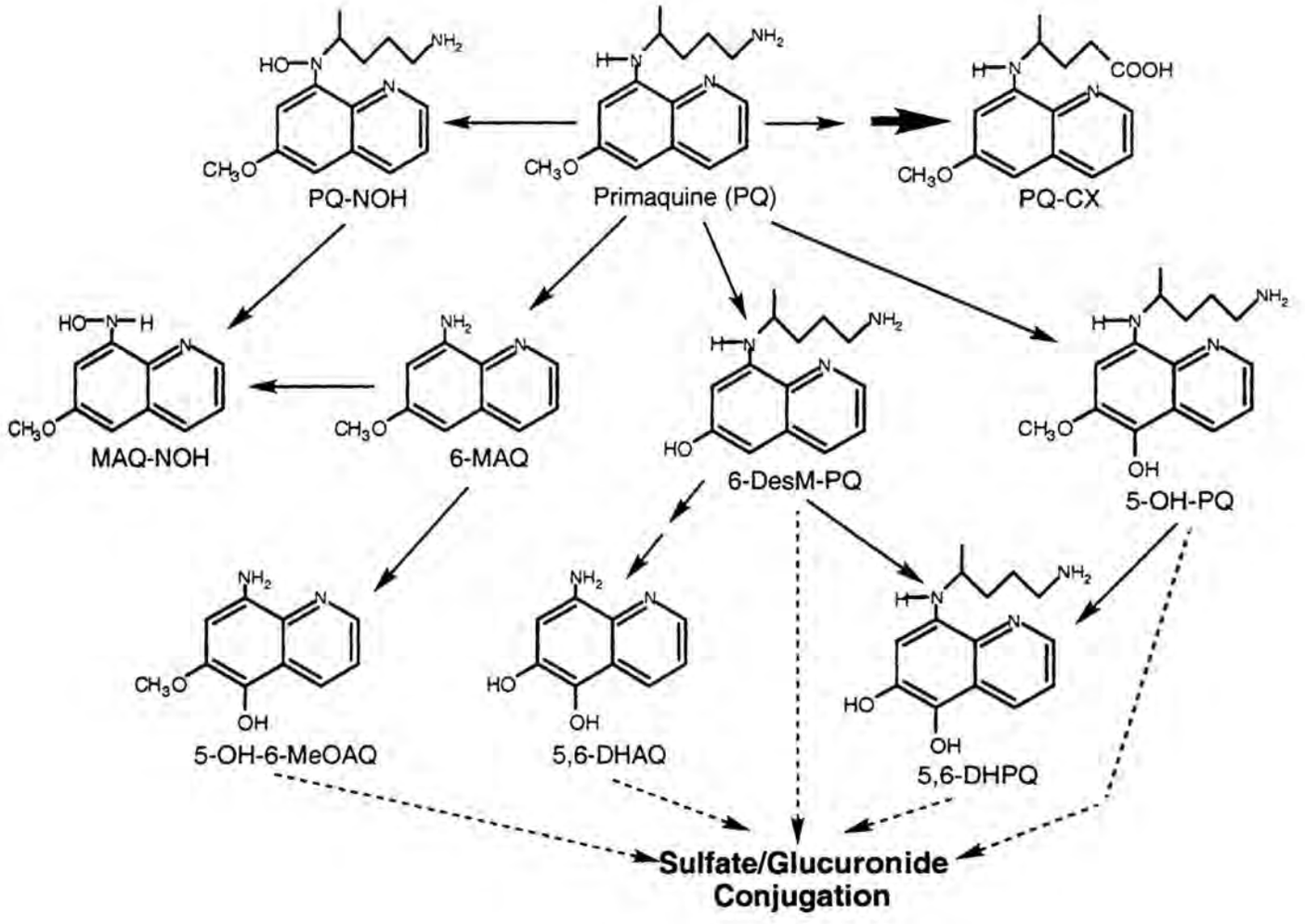

Fig. 1.2. Putative pathways of primaquine metabolism. PQ-NOH, *N*-hydroxyprimaquine; PQ-CX, 8-(3-carboxy-1methylpropylamino)-6-methoxyquinoline; MAQ-NOH, 6-methoxy-8-hydroxylaminoquinoline; 6-DesM-PQ, 6-desmethylprimaquine; 5-OH-PQ, 5-hydroxyprimaquine; 6-MAQ, 6-methoxy-8-aminoquinoline; 5-OH-6-MEOAQ, 5-hydroxy-6-methoxy-8-aminoquinolone; 5,6-DHAQ, 5,6-dihydroxy-8-aminoquinoline; 5,6-

et al., 1984) and rats (Baker et al., 1982; Clark et al., 1984a) in significantly higher concentrations than the parent compound, however, this metabolite was not detected in the urine, indicating further metabolism prior to excretion. The only other known human metabolite of primaquine is the N-dealkylated derivative, 6-methoxy-8-aminoquinoline (6MAQ), which was found in the blood and urine of primaquine treated volunteers (Baty et al., 1975).

Two phenolic metabolites, 5-hydroxyprimaquine (5-OH-PQ) and 6-desmethylprimaquine (6-DesM-PQ) have been identified in the urine of primaquine treated dogs (Strother et al., 1981). In addition to these, 5,6-dihydroxyprimaquine (5,6DHPQ) was detected in the bile of rats during isolated rat liver perfusion studies (Ward et al., 1985). In rat liver microsomal studies, using an ethylchloroformate derivatization technique, Idowu *et. al.* (Idowu et al., 1995) identified multiple metabolites of primaquine including side chain and phenolic oxidation products. However, these investigators did not detect the major rat primaquine metabolite PQ-CX. Conversely, PQ-CX was the only metabolite detected in human liver microsomes (Bangchang et al., 1992).

Oxidative Stress in the Red Blood Cell

The ability of hemoglobin to reversibly bind molecular oxygen and carbon dioxide is essential for tissue oxygenation and removal of carbon dioxide. When bound to hemoglobin, oxygen is known to exist in an equilibrium mixture (fig. 1.3) between oxyhemoglobin and a superoxide-ferri hemoglobin complex (Wittenberg et al., 1970). Formation of this complex is attributed to the capacity of molecular oxygen to acquire

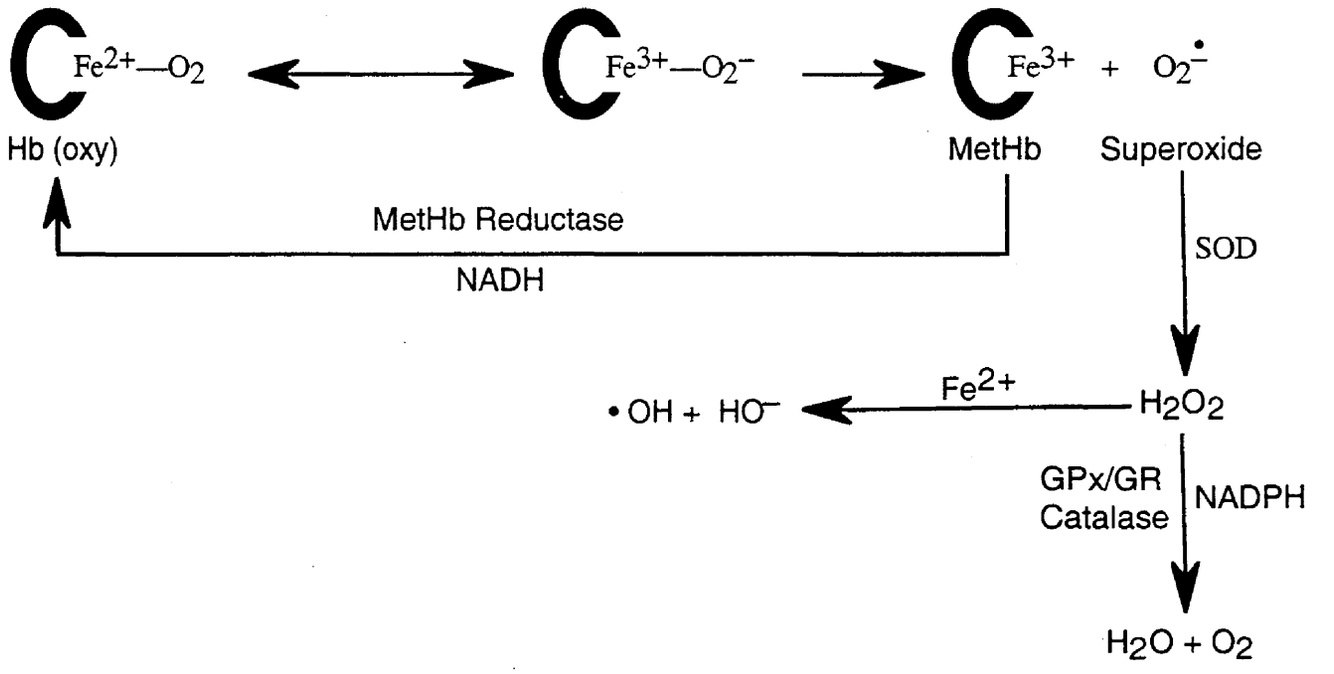

Fig. 1.3. Normal production of active oxygen species within the red cell as a consequence of ferric iron-superoxide anion dissociation from oxyhemoglobin. Hb = oxyhemoglobin; MetHb = methemoglobin; SOD = superoxide dismutase; GPX/GR = glutathione peroxidase/ glutathione reductase.

(from Jollow, 2001)

one of the four unpaired electrons in the outer (d) shell of the heme ferrous iron. When oxygen is released from hemoglobin, this electron is usually returned to the heme iron. However, if the electron remains with the oxygen during its release from hemoglobin, the result is the formation of methemoglobin and a superoxide anion radical (Mansouri and Lurie, 1993). Dissociation of the superoxide-ferri hemoglobin complex occurs constantly in normal red blood cells, and results in the conversion of up to 0.5% of total body hemoglobin to methemoglobin per hour (Chiu et al., 1985).

The red cell contains a number of enzyme systems to cope with the consequences of this oxidative reaction. Thus methemoglobin is readily reduced back to hemoglobin by a NADH-dependant reductase (Passon and Hultquist, 1972), and the superoxide is converted to hydrogen peroxide by superoxide dismutase (Fridovich, 1975). The hydrogen peroxide, in turn, is detoxified by two enzyme systems, catalase and glutathione peroxidase/glutathione reductase (Eaton, 1991) as follows:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{Catalse} 2H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow{GSH\ Px} 2H_2O + GSSG$$

$$GSSG \xrightarrow{GSH\ Red} 2GSH$$

Both catalase and glutathione peroxidase/glutathione reductase enzyme systems require NADPH (Kirkman and Gaetani, 1984). The sole source of this cofactor in the red cell is the first two steps of the hexose monophosphate shunt pathway, the activity of which is controlled by a feedback inhibition of the first enzyme in the sequence, G6PD

by NADPH. Under normal conditions, red cell G6PD is believed to function at less than 3-5% of its maximum capacity (Yoshida and Lin, 1973) and hence the red cell has a significant reserve capacity to enhance production of this protective reductant in the face of increase in oxidative stress.

That this constant low-level oxidant stress has physiological consequences is well illustrated by the fact that human red cells that are deficient in G6PD activity have significantly lower levels of GSH and much shorter life spans (Gaetani et al., 1979). For example, the red cells of individuals with the A- type of G6PD deficiency experience a 20% reduction in red cell life span (Harris and Kellermeyer, 1972) .

It has also long been known that a variety of drugs and other environmental chemicals can enhance the normal level of oxidant stress within the red cell, as indicated by increased cellular levels of methemoglobin and hydrogen peroxide. Chemicals well characterized to have this effect include phenylhydrazine (Cohen and Hochstein, 1964; Goldberg et al., 1976), menadione (Cohen and Hochstein, 1964; Goldberg et al., 1976), and phenylhydroxylamine (Kiese, 1974). More recently, additional active oxygen and other reactive species have been recognized to be formed under these pro-oxidant conditions. Thus superoxide, hydroxyl radical, glutathione and hemoglobin thiyl radicals and ferrylheme have all been detected in red cells exposed to arylhydroxylamines such as phenylhydroxylamine and dapsone hydroxylamine (Maples et al., 1990; Bradshaw et al., 1995; Bradshaw et al., 1997).

Since compounds that enhance oxidant stress within the red cell are hemolytic, and since red cells that are deficient in their capacity to produce the protective reductant, NADPH when faced with such an oxidant stress (i.e., G6PD deficient cells), are uniquely

sensitive to these hemolytic agents, it has long been considered that the hemotoxicity results from damage to the cell inflicted by these oxidant species. However, precisely how these reactive species arise and which of them are responsible for the injury, as well as the identity of the cellular targets, are still unknown and are the subject of much ongoing research.

While it is well recognized the superoxide itself is a relatively non-reactive species in biological systems (Sawyer and Valentine, 1981), in the presence of hydrogen peroxide, this radical can lead to the formation of the highly reactive hydroxyl radical via an iron-catalyzed Fenton reaction (McCord and Day, 1978; Halliwell and Gutteridge, 1992). The hydroxyl radical could further react with cellular sulfhydryl groups to generate thiyl radicals (Maples et al., 1990; Bradshaw et al., 1991; Bradshaw et al., 1995), any of which are capable of inducing oxidative damage to cellular macromolecules, ultimately marking the red cell for premature removal from the circulation by macrophages of the spleen. One problem with this scheme is that ferrous iron is required to catalyze the Fenton reaction, and the ability of hemoglobin to catalyze this reaction is controversial (Gutteridge, 1986; Puppo and Halliwell, 1988; Dong Mao et al., 1994). However, Comporti and colleges have shown that oxidation of hemoglobin to methemoglobin in red cells treated with hemolytic agents such as phenylhydrazine (Ciccoli et al., 1994), divicine (Ferrali et al., 1992), phenylhydroxylamine, and dapsone hydroxylamine (Ciccoli et al., 1999) resulted in the release of diffusible "redox-active" iron.

In addition to hydroxyl radical generation, hydrogen peroxide is also known to react with hemoglobin to form the doubly oxidized hemoglobin species, ferrylhemoglobin (Kanner and Harel, 1985b; Kanner and Harel, 1985a; Harel and Kanner, 1988).

Ferrylhemoglobin (Hb-Fe^{4+}-OH) is a potent oxidant which has been shown to be capable of initiating red cell membrane lipid peroxidation (Kanner and Harel, 1985b; Kanner and Harel, 1985a; Galaris et al., 1990). In addition, ferrylhemoglobin may also react with oxyhemoglobin in a comproportionation reaction to form methemoglobin (Giulivi and Davies, 1990).

$$HbFe^{4+}-OH + HbFe^{2+}-O_2 \longrightarrow 2HbFe^{3+} + O_2$$

In brief, red cells are well known to be subject to an auto-oxidation that results in the formation of methemoglobin and hydrogen peroxide. The red cell is well equipped with oxidant defenses that re-reduce the methemoglobin and detoxify the peroxide. The hemolytic activity of a variety of arylhydroxylamines and other chemicals has been associated with an enhancement of this normally low level oxidant stress and is presumed to result from production of oxidant species at levels and/or rates above the red cells capacity to remove them. At levels above the protective threshold, additional and more toxic oxidant species are thought to arise and it is these species which are considered to be responsible for the cellular injury that causes shortening of the red cell life span by inducing premature splenic sequestration.

Mechanism of Hemotoxicity

While the mechanism by which hemolytic agents damage red blood cells to the extent they are removed prematurely from circulation is poorly understood, it is believed that these agents enhance the process of normal reactive oxygen species formation such that the antioxidant defenses in the red cell are overwhelmed resulting in an "oxidative stress" (Beutler, 1959; Tarlov et al., 1962; Miller and Smith, 1970; Gordon-Smith and White,

1974; Carrell et al., 1975). Observations that support this idea include: 1) a decrease in reduced red blood cell glutathione accompanies a hemolytic episode, 2) hemoglobin oxidation to methemoglobin and the formation of Heinz bodies (denatured hemoglobin) following exposure to hemolytic agents, and 3) an enhanced sensitivity to these drugs in individuals that have a decreased ability to generate adequate levels of NADPH (i.e., G6PD-deficient people) when presented with an oxidative challenge. It was also realized during these early mechanistic studies that many of these hemolytic agents were not strong oxidizing or reducing agents and did not induce hemolytic damage when incubated with red cells *in vitro* at physiological relevant concentrations (Fraser and Vesell, 1968; Miller and Smith, 1970; Fletcher et al., 1988). Thus the concept developed that many of these hemolytic compounds were metabolized *in vivo* to more redox active species that were responsible for oxidizing critical cellular constituents and initiating the premature removal of the red cells from circulation (Tarlov et al., 1962; Fraser and Vesell, 1968; Beutler, 1971).

In this regard, investigators have examined the possible role primaquine phenolic derivatives may play in the parent compound toxicity. 5-OH-PQ, 5,6-DHPQ, and 5,6-DHAQ, have been shown *in vitro* to deplete erythrocytic GSH and oxidize oxyhemoglobin to methemoglobin in both normal and G6PD deficient red cells (Fraser and Vesell, 1968; Agarwal et al., 1988; Fletcher et al., 1988). It is proposed that these compounds play a role in primaquine-induced hemotoxicity by redox cycling within the red cells to their corresponding quinone/ quinone-imine derivatives consequently forming reactive oxygen species that oxidize critical cellular constituents terminally damaging the cell (Vasquez-Vivar and Augusto, 1992; Vasquez-Vivar and Augusto, 1994). It is of

interest that phenolic metabolites of the hemolytic agent aniline were also found to oxidize hemoglobin *in vitro*, however, they were not hemolytic *in vivo* in rats (Harrison and Jollow, 1986; Harrison and Jollow, 1987). These metabolites although formed in the liver, are quickly cleared by conjugation reactions and therefore do not contribute to the parent compound toxicity.

Although it is widely accepted that drug-induced hemolytic anemia results from oxidative damage to the red cells, the mechanism in which these agents impose this damage is not known. However, some evidence is available to suggest that more than one mechanism may exist. An elegant study conducted in 1966 on inmates in the Illinois State Penitentiary (Degowin et al., 1966), illustrated in figure 1.4, demonstrated that when the hemolytic drug dapsone was administered to G6PD-normal and G6PD-deficient (A-) volunteers, there was moderate induction of hemolytic anemia in normal individuals and about a 2-fold increase in sensitivity towards dapsone-induced hemolytic anemia in G6PD-deficient individuals. In marked contrast, primaquine had little hemolytic activity in normal individuals, however, A- G6PD deficiency was associated with a 30-fold increase in sensitivity to primaquine hemotoxicity. While interpretation of the data remains unclear, it argues strongly for marked variations in the response of red cells to hemolytic metabolites of these two drugs.

Role of Hydroxylamines in Drug-Induced Hemolytic Anemia

Arylhydroxylamines are known to mediate the hemotoxic side-effects of hemolytic agents such as aniline, dapsone, propanil, and phenacetin (Harrison and Jollow, 1986; Grossman and Jollow, 1988; Jensen and Jollow, 1991; McMillan et al., 1991). It is

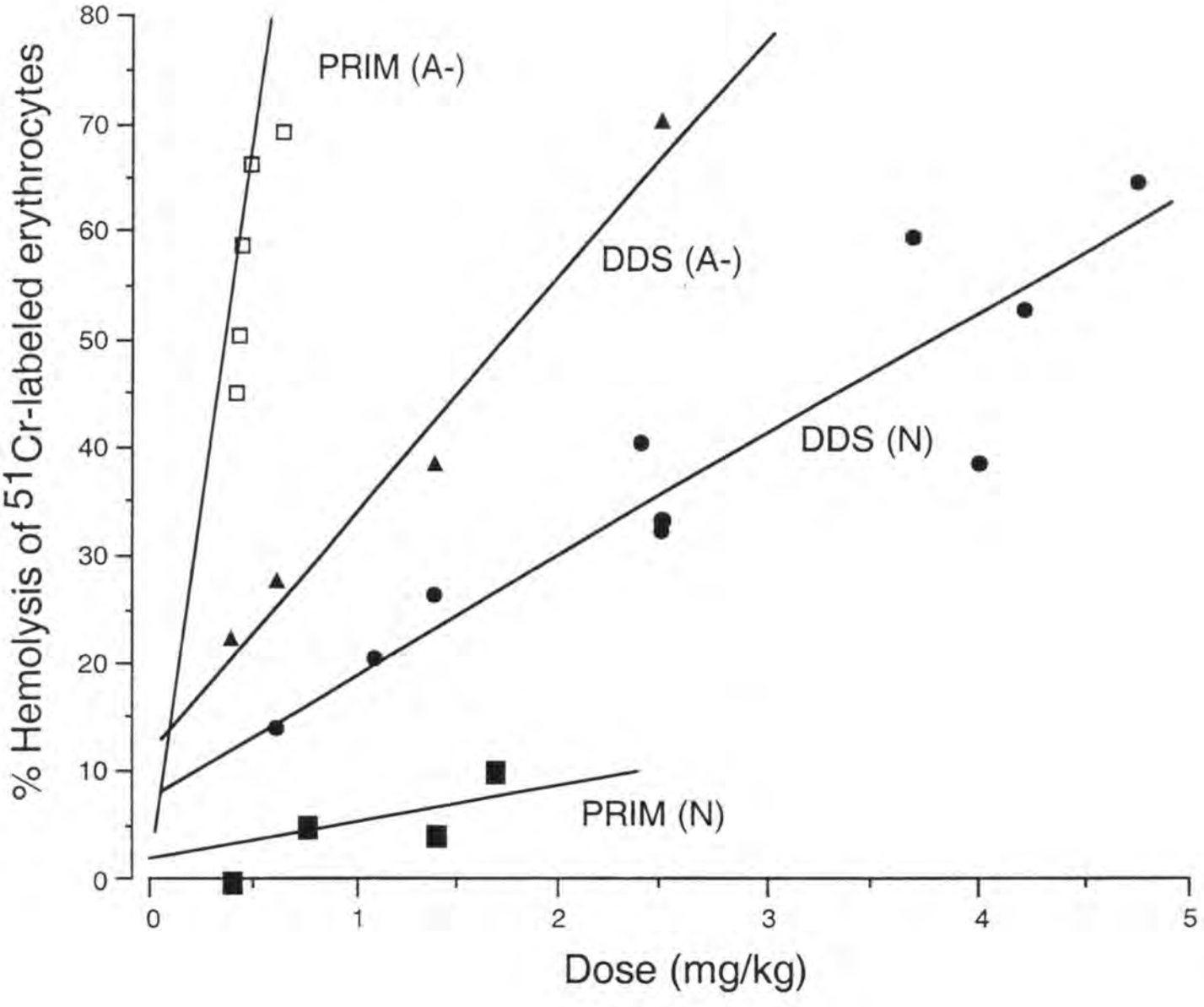

Fig. 1.4. Effect of G6PD (A-) deficiency on the hemolytic activity of dapsone and primaquine in volunteers. PRIM (N) = primaquine in normal individuals, PRIM (A-) = primaquine in A- G6PD-deficient individuals, DDS (N) = dapsone in normal individuals, DDS (A-) = dapsone in A- G6PD-deficient individuals.

(from Degowin, 1966)

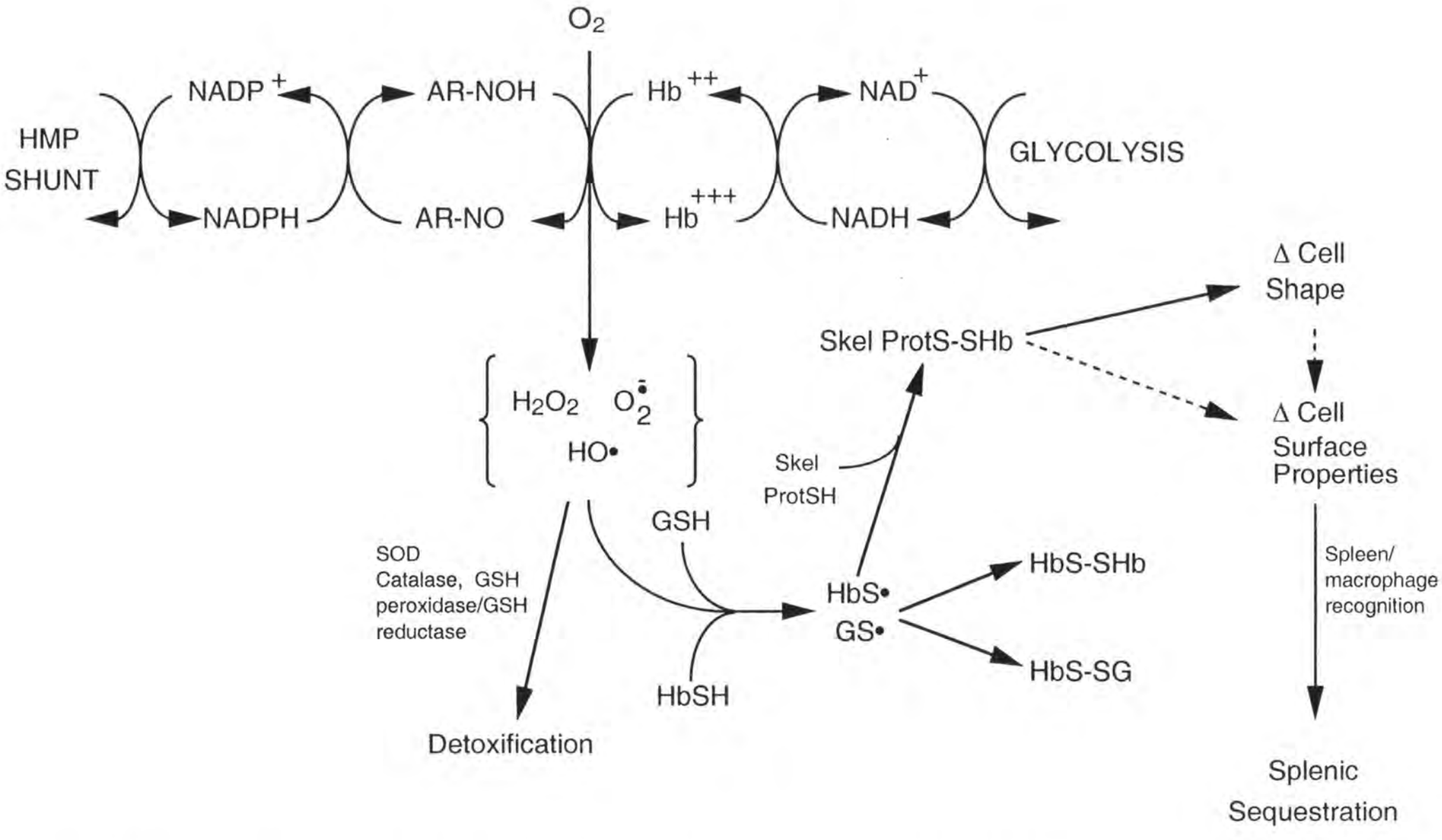

Fig. 1.5. Working Hypothesis for Arylhydroxylamine-induced Hemolytic Activity. AR-NOH = arylhydroxylamine; Hb^{++} = ferrohemoglobin; Hb^{+++} = ferrihemoglobin; HMP = hexose monophosphate shunt; SOD = superoxide dismutase

(from Grossman, 1992)

believed that these metabolites evoke an oxidative stress within the red cell through a cyclic oxidation-reduction reaction (fig. 1.5) that involves oxyhemoglobin and molecular oxygen and yields the nitrosoarene, methemoglobin, and partially reduced forms of oxygen, respectively (Rostorfer and Cormier, 1957; Kiese, 1974). Based on EPR spin trapping experiments, these reactive oxygen species have been shown to further generate more reactive hydroxyl radicals and glutathione and hemoglobin thiyl radicals (Maples et al., 1990; Bradshaw et al., 1995; Bradshaw et al., 1997) that are capable of critically damaging the red cell. In addition, this reaction is also believed to generate a phenylnitroxide radical intermediate through the one-electron oxidation of phenylhydroxylamine (Maples et al., 1990). The nitrosoarene is subsequently reduced back to the hydroxylamine by NADPH-dependent reductase and is available to oxidize another equivalent of hemoglobin. Thus methemoglobin and activated oxygen species are produced in greater than stoichiometric amounts resulting in an oxidant stress within the red cell.

Cellular Targets

Although the precise internal lesion that ultimately marks a red cell for sequestration is not known, four major targets for oxidative attack have been proposed:

1. **Hemoglobin.** As mentioned previously, several known and putative primaquine phenolic metabolites including 5-OH-PQ, 5,6-DHPQ, and 5,6-HAQ have been shown to oxidize hemoglobin to methemoglobin. It is known that heme dissociates more readily from methemoglobin (Itano, 1970; Gutteridge, 1986) after which free globin precipitates easily. Denatured

hemoglobin is thought to attach to the plasma membrane to form Heinz bodies (Jacob, 1970). Heinz bodies have been reported to occur in red cells isolated from primaquine treated patients (Beutler et al., 1954). The presence of Heinz bodies may mechanically impede the passage of erythrocytes through the spleen (Lubin and Desforges, 1972; Weinstein et al., 1975), alter the adjacent membrane leading to splenic recognition and sequestration, or lead to splenic entrapment by inducing spherocyte formation due to splenic abstracting of the Heinz bodies along with membrane lipids known as a "pitting" process (Rifkind and Danon, 1965). Alternatively, heme oxidation may result in the release of iron in a free and diffusible form, which may catalyze the formation of hydroxyl radicals via the Fenton reaction (Ferrali et al., 1992; Ciccoli et al., 1994; Ciccoli et al., 1999). Hydroxyl radicals are highly reactive, and can attack a number of intracellular sites, including membrane lipids and proteins.

2. **Sulfhydryl groups.** Changes in cellular sulfhydryl status have long been associated with the premature sequestration of red blood cells. As demonstrated by Jacob *et. al.*, titration of erythrocyte sulfhydryl groups with *N*-ethylmaleimide or *p*-chloromercuribenzoate causes the cells to be rapidly taken up by the spleen (Jacob and Jandl, 1962). In addition sulfhydryl oxidation may promote the formation of mixed disulfides between hemoglobin and glutathione and between hemoglobin and other cellular proteins. It has been suggested that these interactions cause a change in the tertiary/ quaternary structure of hemoglobin facilitating its precipitation to

form Heinz bodies resulting in splenic removal as described above (Allen and Jandl, 1961; Kosower et al., 1977).

3. **Membrane proteins.** The crucial role of the membrane skeletal proteins in maintaining the structure of the red cell is well established (Cohen, 1983; Marchesi, 1985). Oxidative cross-linking spectrin molecules to form high molecular weight aggregates using oxidants such as diamide is thought to diminish the lateral mobility of transmembrane proteins (Haest et al., 1977), thus "fixing" the membrane into a rigid structure that could become trapped in the microvasculature of the spleen. Alternatively, as detailed below, alterations in the form of hemoglobin disulfide adducts to the integral membrane protein band 3 may signal premature removal of the red cell from the circulation.

4. **Membrane lipids**. Free radical-induced membrane lipid peroxidation has been frequently cited as possible mechanism of toxicity (Stocks and Dormandy, 1971; Rasbridge and Scott, 1973; Goldstein et al., 1980; Lubin and Chiu, 1982). Crosslinking of membrane lipids with each other and/or with membrane proteins could result in decreased membrane fluidity (Dobretsov et al., 1977; Hochstein and Jain, 1981), causing erythrocyte retention in the spleen. Lipid peroxidation may also cause the externalization of phosphatidyl serine (Jain, 1984), with a resultant decrease in membrane deformability or of enhanced adherence to macrophages (Tanaka and Schroit, 1983; Wagner et al., 1985; McEvoy et al., 1986; Boas et al., 1998).

Senescence and Splenic Sequestration

Normal red blood cells are predominantly removed from the circulation intact by resident macrophages in the spleen (Azen and Schilling, 1963; Tizianello et al., 1968). It is thought that oxidative drugs such as primaquine inflict injury within the red cell that results in premature recognition of the cells as "aged", thus provoking their removal via the normal sequestration pathway in the spleen. Intravascular lysis and hepatic sequestration are believed to play a role in erythrocyte removal when the hemolytic insult is severe and a very high number of red cells are damaged (Rifkind, 1966; Cooper and Jandl, 1972).

The mechanism(s) in which splenic macrophages specifically recognize aged or injured red cells to be phagocytized is still not understood. However, a significant amount of data has been generated in support of three separate hypotheses (fig 1.6) (for review see Bratosin et al., 1998). The first suggests a role for the enzymatic removal of sialic acid in unmasking carbohydrate epitopes, particularly β-galactosyl residues on glycophorin A. Using FITC-labeled lectins, investigators have shown an increase in desialytion of glycophorins with red cell age (Sharon and Fibach, 1991; Fibach and Sharon, 1994). In addition, erythrophagocytosis of red cells treated with neuraminidase to remove sialic acid residues is inhibited with β-galactosides. A β-galectin on the macrophage membrane has been implicated in recognizing the β-galactosyl residues on aged red cells and initiating erythrophagocytosis. However, Aminoff *et. al.* have also shown that immunoglobulins may play a role in the recognition process (Alderman et al., 1981; Aminoff et al., 1991).

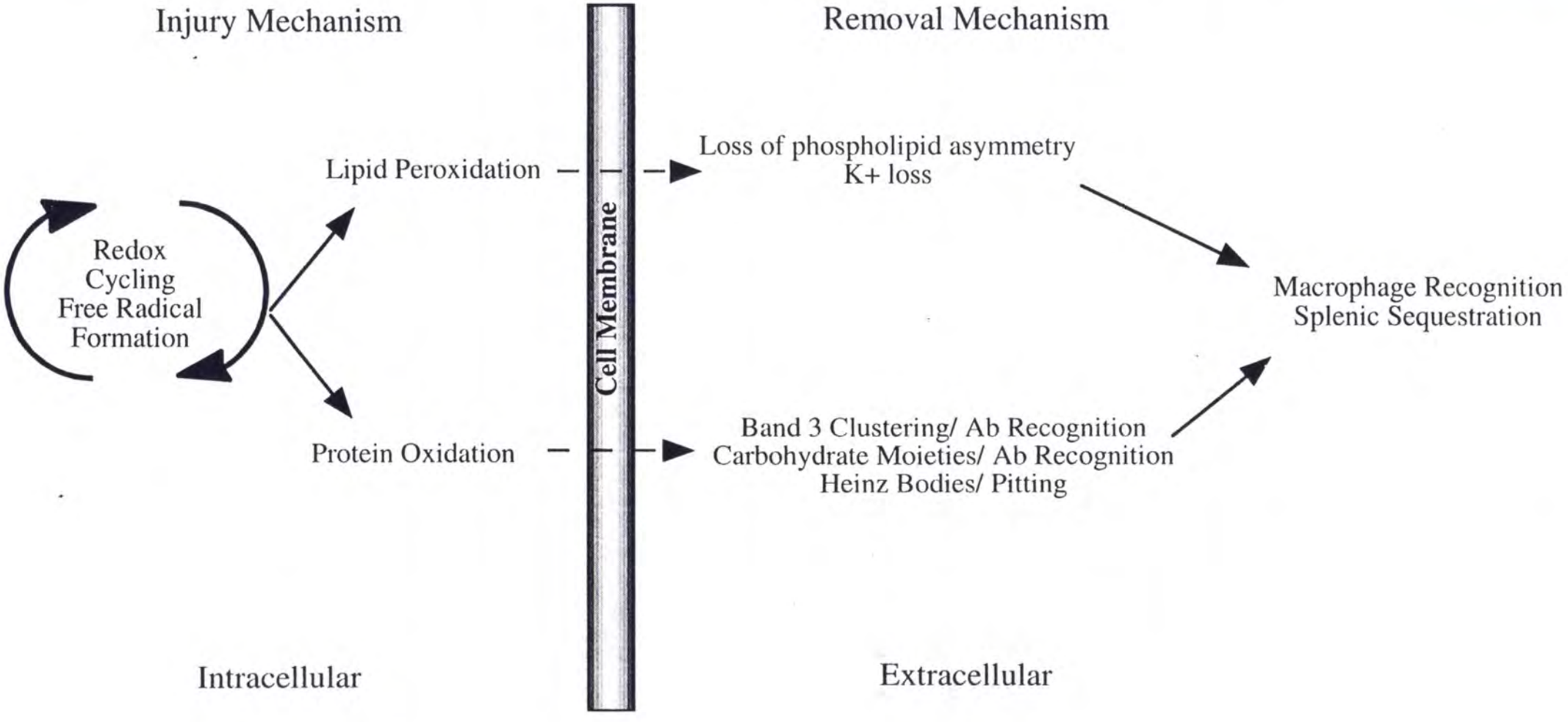

Fig. 1.6. Conceptual Relationships Between Injury and Removal of Red Cells

A second possible mechanism in which to elicit macrophage ingestion of damaged or aged erythrocytes includes disruption of the normal membrane phospholipid asymmetrical distribution, in particular phosphatidyl serine (PS). Normally PS is maintained predominantly in the inner leaflet of the plasma membrane by the energy dependent transporter aminophospholipid translocase (i.e., flippase). Dissipation of the PS transbilayer gradient has been shown to play an important role in cell-cell recognition processes including macrophage recognition of apoptotic (Fadok et al., 1992; Bratton et al., 1997) and tumorigenic cells (Conner et al., 1991; Utsugi et al., 1991). Of interest, PS externalization is also known to drastically enhance macrophage phagocytosis of red blood cells *in vitro* (Tanaka and Schroit, 1983; McEvoy et al., 1986) as well as red cell clearance *in vivo* (Schroit et al., 1985). In addition, aged, sickled, and uremic red cells (Conner et al., 1994; Boas et al., 1998) have been shown to contain PS in their outer membranes and to bind to macrophages more readily (Tait and Gibson, 1994; Wood et al., 1996) than normal erythrocytes. It was hypothesized that PS exposure triggers macrophage recognition and ingestion of aged or damaged erythrocytes.

Finally, the unmasking of a peptidic antigen that leads to opsonization by circulating autologous antibodies may signal a red cell for sequestration. A number of studies have suggested a key role for hemoglobin binding to cytoskeletal proteins in creating this antigenic site. In this regard, early observations by Morrison *et. al.* (Morrison et al., 1983) of aged red cells included an increase in hemoglobin association with the cell membranes prior to their sequestration. Low and colleagues later reported that hemoglobin avidly binds the cytoplasmic region of the integral membrane protein band 3 and stimulates its aggregation (Waugh and Low, 1985; Waugh et al., 1986). In addition, these investigators

established that clustering of band 3 induced IgG opsonization and subsequent macrophage ingestion (Low et al., 1985; Turrini et al., 1991). These data lead to the hypothesis that as the red cells age or are damaged, hemoglobin binds to band 3 and initiates the formation of protein aggregates resulting in the more stable binding of autologous antibodies. Once a critical density of bound antibody is reached, complement binds and the cells are recognized by immunoglobulin and complement receptors on the macrophages and phagocytized.

Rationale and Specific Aims

While metabolites of primaquine have long been known to mediate the parent compound hemotoxic side effects (Tarlov et al., 1962; Fraser and Vesell, 1968), the metabolic profile of primaquine remains poorly defined. The reasons for this are multi-factorial beginning with primaquine having a molecular structure with multiple possible oxidation sites that gives rise to a large number of known and proposed metabolites (fig. 1.2). Moreover, these metabolites are highly unstable in biological systems (Strother et al., 1984; Idowu et al., 1995). Adding to the complexity of this problem, Lee et al. (Lee et al., 1981) have demonstrated substantial species variations in sensitivity towards primaquine. For example, primaquine hemotoxicity has been particularly difficult to express in rats after both acute and chronic exposure (Bolchoz unpublished results, Lee et al., 1981). Thus classical pharmacokinetic studies to identify potentially hemotoxic metabolites and determine the relative contribution to primaquine hemotoxicity are extremely difficult.

The long-term goal of our laboratory is to understand the mechanism underlying primaquine-induced hemolytic anemia. Therefore identification of the potentially hemotoxic metabolite(s) is crucial. As previous studies have suggested that drug-induced hemolytic anemia is mediated by oxidative stress, the experimental approach is to identify redox active metabolites of primaquine that are potentially hemolytic. We intend to examine the metabolic formation of these derivatives, determine if they are hemolytic, and if so, to characterize the "pattern" of damage (i.e., the ratio of lipid to protein oxidation, change in morphology, effect on cellular sulfhydryl status, nature of radical formation) induced in rat red cells by these agents under hemolytic conditions.

In addition to facilitating the effort to identify the hemotoxic metabolite(s) of primaquine, these studies will contribute significantly to the extensive efforts to elucidate the mechanism of drug-induced hemolytic anemia. In examining the extent to which these hemolytic agents induce the multiple intracellular lesions mentioned above, we can begin to assess the significance of these effects in the premature removal of red cells from the circulation.

In contributing to this overall goal, the present studies examine the possible role of 6-methoxy-8-hydroxylaminoquinoline (MAQ-NOH) in primaquine-induced hemolytic anemia. In doing so, we examined MAQ-NOH metabolic formation, hemolytic activity, and mechanism of inducing hemolytic anemia. The reasons for starting with the N-hydroxy type metabolite are two fold. Previous studies in our laboratory have shown that the N-hydroxylated metabolites of hemolytic agents such as aniline, dapsone, propanil, and phenacetin are responsible for the parent compound hemotoxicity (Harrison and Jollow, 1986; Grossman and Jollow, 1988; Jensen and Jollow, 1991; McMillan et al.,

1991). Secondly, 6-methoxy-8-aminoquinoline (6-MAQ), a logical precursor for MAQ-NOH formation, is one of two known human primaquine metabolites (Baty et al., 1975). Thus the following hypothesis and specific aims were developed:

Hypothesis: The primaquine metabolite, MAQ-NOH, induces hemolytic anemia in the rat by initiating an oxidative stress within the red cell that alters the red cell membrane integrity.

Specific Aim 1. 1) To establish if 6MAQ is metabolized to MAQ-NOH using rat and human microsomes. 2) To determine the potency of MAQ-NOH with respect to hemolytic and methemoglobinemic activity.

Specific Aim 2. To examine the morphological and oxidative effects of MAQ-NOH in rat erythrocytes in the presence and absence of GSH focusing on lipid peroxidation and membrane skeletal protein damage.

Specific Aim 3. To identify the radical species formed in response to MAQ-NOH in rat erythrocytes under hemolytic conditions.

CHAPTER 2

Primaquine-induced Hemolytic Anemia: Formation and Hemotoxicity of the Arylhyroxylamine Metabolite 6-Methoxy-8-hydroxylaminoquinoline

Introduction

Malaria is considered to be the most widespread parasitic infection in the world, with nearly one third of the world population threatened by *Plasmodium sp.* infections (Miller, 1997). Primaquine, an 8-aminoquinoline drug, has been an important antimalarial agent for over 40 years because of its unique effectiveness against exoerythrocytic forms of the parasite. Primaquine is currently the only drug capable of treating the persistent liver stages of *P. vivax* and *P. ovale* responsible for relapsing malaria (Tracy and Webster, 1996). Furthermore, the importance of primaquine has grown recently due to development of resistance against other antimalarial drugs, and because of its potential for use against opportunistic *Pneumocystis carinni* pneumonia in AIDS patients (Kantor, 1992).

Despite its broad antiparasitic activity, primaquine therapy is limited by its capacity to induce a Heinz-body anemia, particularly in individuals with deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity (Dern et al., 1955; Degowin et al., 1966). Deficiency in erythrocytic G6PD has been shown to confer resistance to infection by the malarial parasite, which presumably accounts for the high incidence of G6PD deficiency in areas endemic for malaria (Beutler, 1978). Early mechanistic studies established that 1) primaquine hemotoxicity was due to metabolite(s) of the drug, rather than the parent compound; 2) glutathione became oxidized to glutathione disulfide and was lost from the red cell; 3) hemoglobin became denatured and formed large insoluble aggregates that were attached to the inner surface of the red cell membrane (i.e., Heinz bodies); and 4) the hemolytic activity was associated with an accelerated removal of the damaged (but intact) red cells from the circulation by the spleen and liver (for review, see Beutler, 1969). The

identity of the hemotoxic metabolite(s), however, was not determined, and the mechanism underlying the hemolytic response to primaquine remains unknown.

Phenolic metabolites of primaquine have long been suggested as candidate hemotoxic species (Tarlov et al., 1962). These oxidation products have been identified as metabolites of primaquine in the biological fluids of experimental animals (Strother et al., 1981; Strother et al., 1984; Idowu et al., 1995), and they have been observed to cause oxidative damage to normal and G6PD-deficient erythrocytes (Strother et al., 1984; Agarwal et al., 1988; Fletcher et al., 1988). However, phenolic metabolites have not been detected in humans after administration of primaquine (Baty et al., 1975; Mihaly et al., 1984) or in primaquine-treated human liver microsomes (Bangchang et al., 1992).

An alternate hypothesis is that the hemotoxicity of primaquine is associated, in part or in whole, with an arylhydroxylamine metabolite. Primaquine is known to undergo N-dealkylation in humans to yield 6-methoxy-8-aminoquinoline (6-MAQ) (Baty et al., 1975). In previous studies with other arylamines, such as aniline and dapsone, we have observed that their N-hydroxy metabolites are direct-acting hemotoxicants and are responsible for the hemolytic activity of the parent compounds (Harrison and Jollow, 1986; Grossman and Jollow, 1988). It is thus plausible that 6-MAQ could be converted to its N-hydroxy analog (fig. 2.1), and that this metabolite could be capable of inducing methemoglobinemia and hemolytic damage.

To investigate whether an arylhydroxylamine metabolite could be involved in primaquine hemotoxicity, we have synthesized the putative N-hydroxy metabolite, 6-methoxy-8-hydroxylaminoquinoline (MAQ-NOH), and have examined the capacity of rat and human liver microsomes to form this metabolite. In addition, we have assessed the ability of MAQ-NOH to induce methemoglobin formation and hemolytic damage in rat erythrocytes. We report that MAQ-NOH was formed in both rat and human liver microsomes, and that MAQ-NOH is a direct-acting methemoglobinemic and hemolytic agent in rats. These results suggest that N-dealkylation of primaquine followed by N-

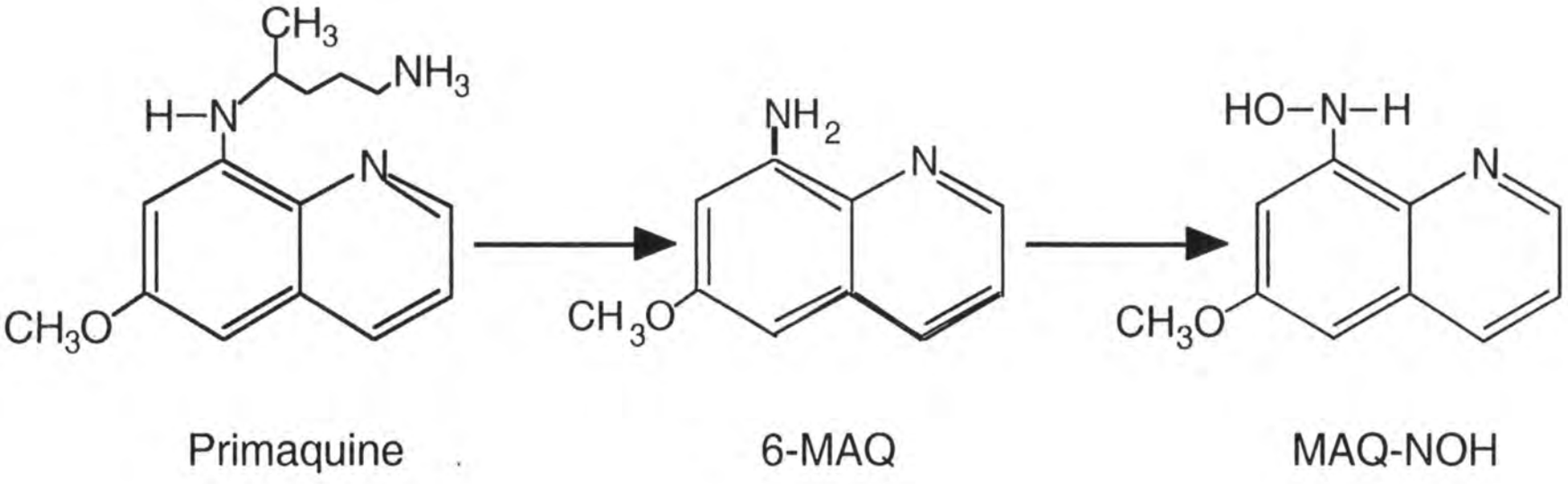

Fig. 2.1. Proposed pathway of primaquine metabolism to the hemotoxic arylhydroxylamine metabolite.

oxidation of 6-MAQ may be a contributing pathway for the expression of primaquine hemotoxicity.

Materials and Methods

Chemicals and Materials

6-Methoxy-8-nitroquinoline, platinum oxide, stannous chloride and granular tin were purchased from Aldrich Chemical Co. (Milwaukee, WI). GSH, $NADP^+$, and DMSO-d_6 were obtained from Sigma Chemical Co. (St. Louis, MO). $Na_2{}^{51}CrO_4$ in sterile saline (1 mCi/ml, pH 8) was purchased from New England Nuclear (Billerica, MA). Male Sprague-Dawley rat and pooled human liver microsomes were purchased from In Vitro Technologies (Baltimore, MD). All other chemicals were of the best commercially available grade.

Animals

Male Sprague-Dawley rats (75-100 g) were obtained from Harlan Laboratories (Indianapolis, IN), and were maintained on food and water *ad libitum*. Animals were acclimated for 1 week to a 12-hr light-dark cycle prior to their use. Blood from the descending aorta of anesthetized rats was collected into heparinized tubes and washed three times with isotonic phosphate-buffered saline supplemented with glucose (PBSG) (110 mM NaCl, 20 mM Na_2HPO_4, 4 mM KH_2PO_4, 10 mM D-glucose, pH 7.4) to remove the plasma and buffy coat. The cells were resuspended in PBSG and used the same day they were collected.

Synthesis of 6-MAQ and MAQ-NOH

6-MAQ was synthesized previously in our laboratory by reduction of 6-methoxy-8-nitroquinoline to the amine using tin/stannous chloride as described previously (Furniss et al., 1989). The crude product was recrystallized from methanolic HCl as the hydrochloride salt. To determine if the compound was still intact and pure, an NMR spectrum was

obtained (fig. 2.2) with chemical shifts (in ppm referenced to the solvent) at 3.86 (s, 3H, 6-OCH_3), 6.92 (d, J=2.5 Hz, 1H, H_5), 6.96 (d, J=2.5 Hz, 1H, H_7), 7.74 (dd, J=4.8 Hz, J=8.4 Hz, 1H, H_3), 8.59 (d, J=8.4 Hz, 1H, H_4), 8.77 (dd, J=1.5 Hz, J=4.7 Hz, 1H, H_2); the 8-amino protons were not observed.

MAQ-NOH was synthesized as previously described (Allahyari et al., 1984). Breifly, 40 mg of platinum oxide in 13.33 ml of ethyl acetate was reduced for in a Parr hydrogenator for 5 min under 12 psi of hydrogen. Next, 4 grams of 6-methoxy-8-nitroquinoline in 93.3 ml of ethyl acetate was added to a suspension and reduced for 1 hour at 31 psi. The reaction was cooled in the freezer overnight and the precipitate was recrystallized once from acetone. HPLC analysis of the product showed <1% residual 6-methoxy-8-nitroquinoline. Mass spectral analysis (fig. 2.3) revealed a molecular ion (M^+) at *m/z* 191, and a predominant fragment ion at *m/z* 175. MS/MS analysis of the M^+ revealed fragment ions at *m/z* 176 (M^+ –15), 173 (M^+ –18), and 162 (M^+ –29). The NMR spectrum of MAQ-NOH (fig. 2.4) showed chemical shifts at 3.84 (s, 3H, 6-OCH_3), 6.69 (d, J=2.7 Hz, 1H, H_5), 6.76 (d, J=2.7 Hz, 1H, H_7), 7.43(dd, J=4.2 Hz, J=8.3 Hz, 1H, H_3), 8.13 (dd, J=1.6 Hz, J=8.3 Hz, 1H, H_4), 8.56 (dd, J=1.7 Hz, J=4.2 Hz, 1H, H_2), 8.67 (s, 1H, -NH), 8.79 (s, 1H, -NOH). DQ-COSY analysis of the cross peaks confirmed the assignment of the aromatic ring protons. The synthetic compounds were stable under argon at –80°C.

HPLC Analysis

Chromatography was performed on a Waters HPLC system (Waters Assoc., Milford, MA) consisting of a Model 6000A pump, a Rheodyne injector (20 μl loop) and a 250 mm Altech Platinum EPS C_{18} reverse-phase column. 6-MAQ was eluted with 35% acetonitrile in water at a flow rate of 1.2 ml/min, and was detected on a Waters model 481 UV-Vis variable wavelength detector set at 280 nm. MAQ-NOH was eluted with 40% methanol in water containing 0.1 % trifluoroacetic acid and 10 mM ammonium acetate at a flow rate of

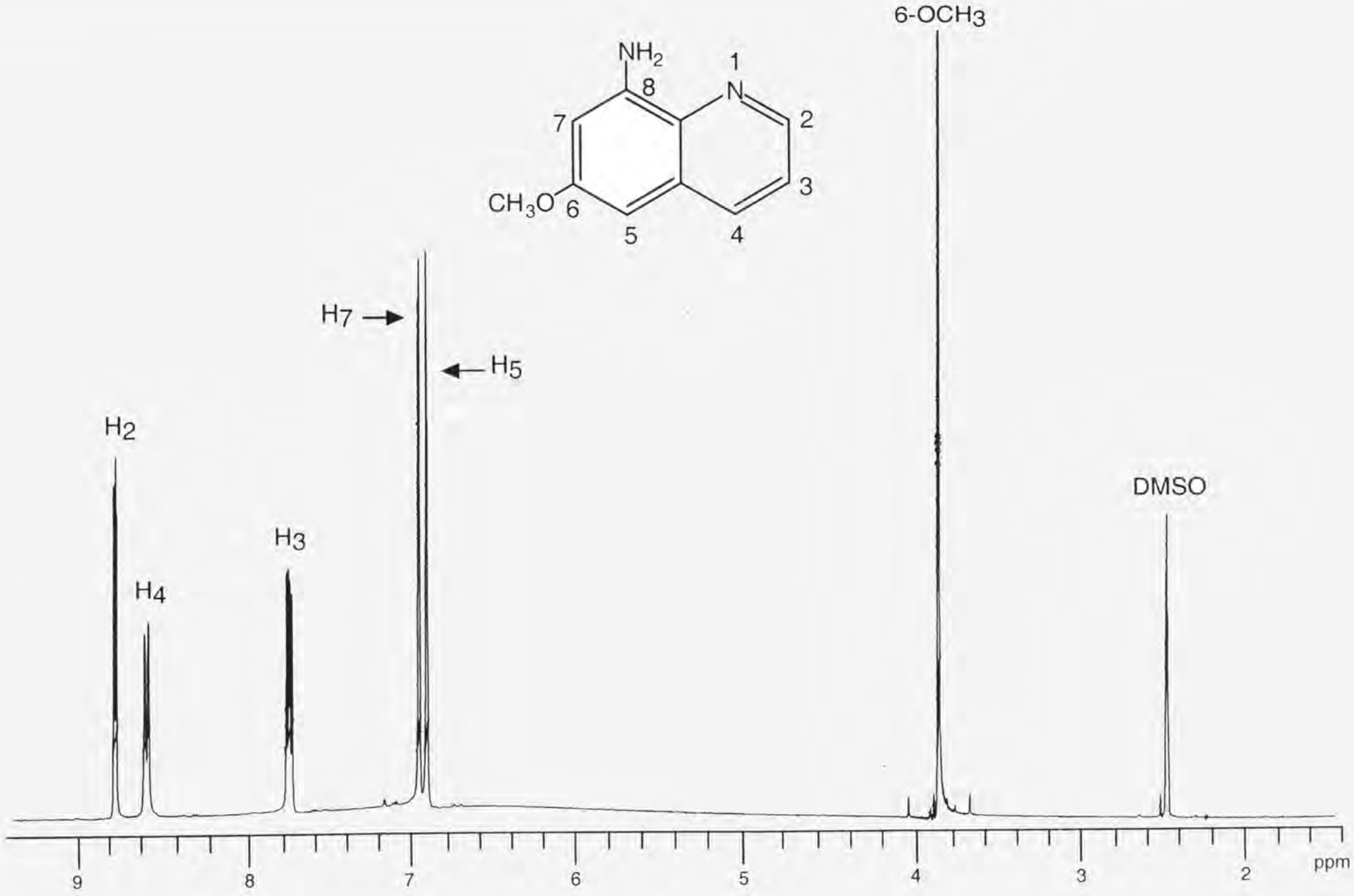

Fig. 2.2. NMR spectrum of synthetic 6-MAQ

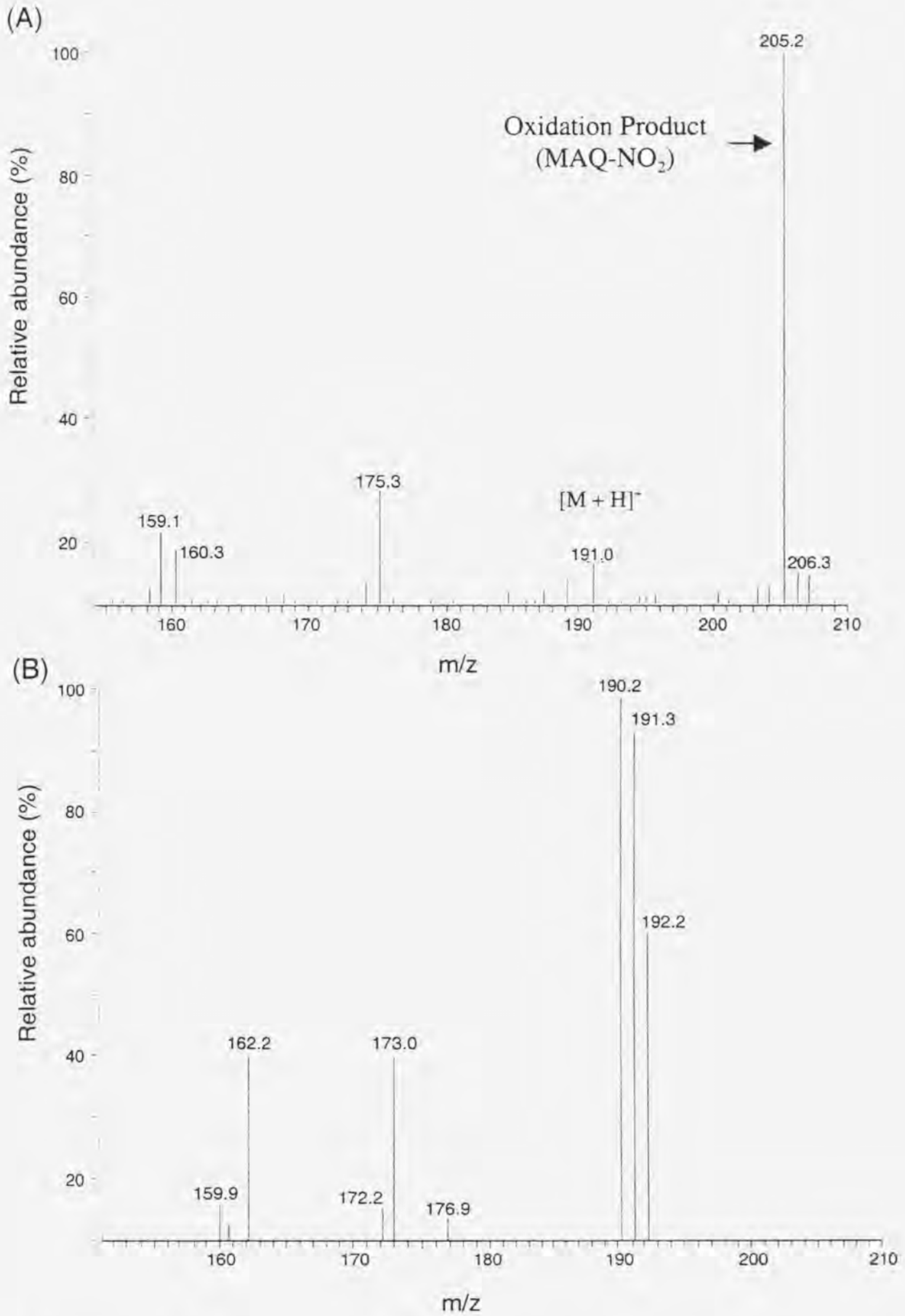

Fig. 2.3. (A) ESI mass spectrum of synthetic MAQ-NOH. (B) ESI MS/MS of the parent ion (*m/z* 191).

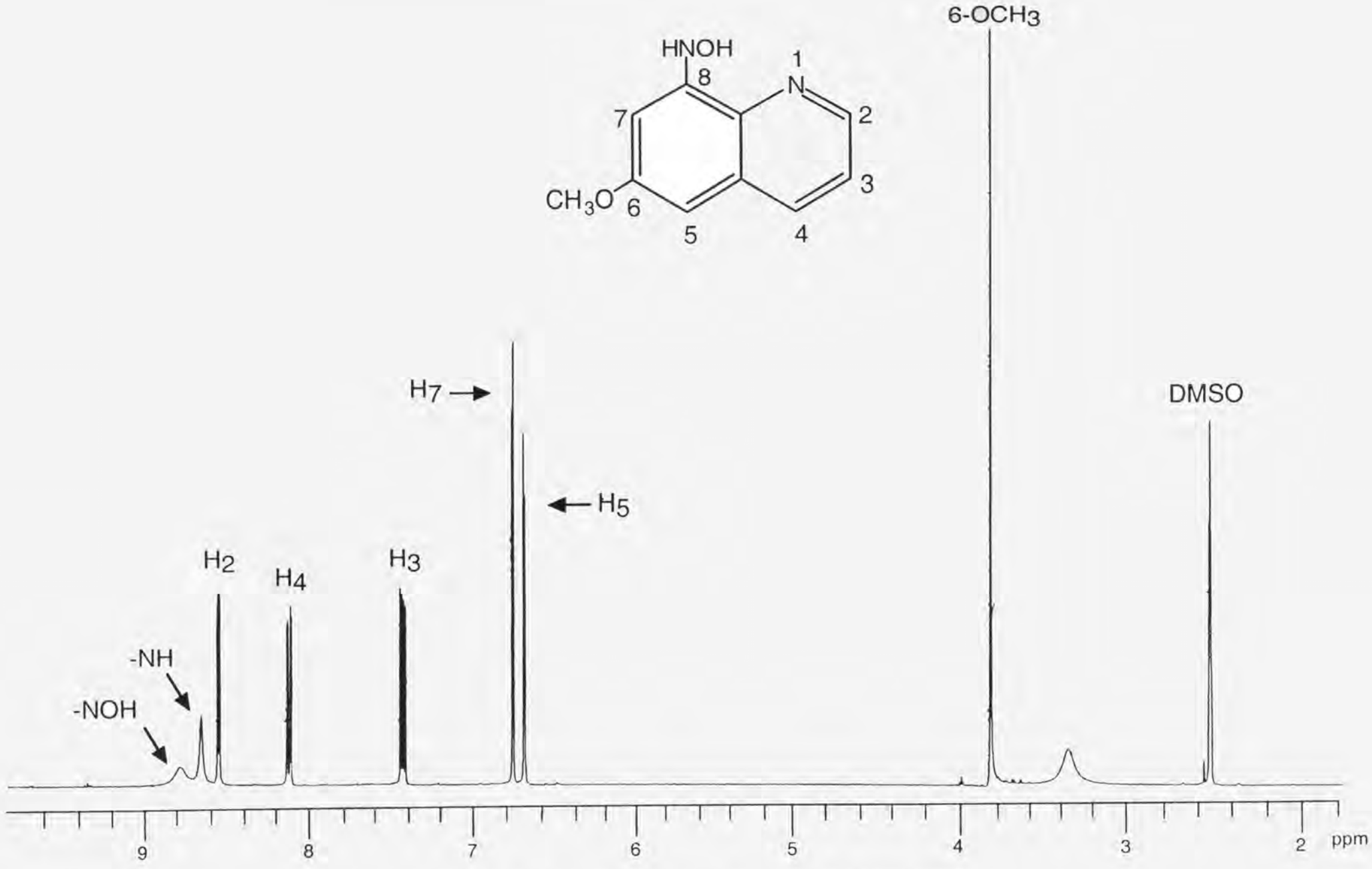

Fig. 2.4. NMR spectrum of synthetic MAQ-NOH

1.5 ml/min, and was detected using either 1) a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy carbon working electrode (oxidation mode, +0.35 volts) and a Ag/AgCl reference electrode; or 2) a Waters Millennium model 996 photodiode array detector.

NMR Spectroscopy and Mass Spectrometry

Mass spectral analysis was performed on a Finnigan LCQ Quadrapole ion trap tandem mass spectrometer. The instrument was operated in positive-ion mode with electrospray ionization (ESI) needle voltage, 4.2 kV; ESI capillary temperature, 200°C; isolation width, 2 amu; scan range 100-500 amu. MS/MS data was simultaneously acquired for the selected parent ion (*m/z* 191). Helium was used to fragment the ion with a collision parameter set to 35. Proton NMR spectra were acquired on a Varian VXR 400 spectrometer operating at 400 MHz with conventional quadrature detection.

Microsomal metabolism of 6-MAQ

6-MAQ (1 mM) was incubated with male Sprague-Dawley rat and pooled human liver microsomes (1.0 mg protein/ml) in 100 mM phosphate buffer pH 7.4. The reaction was initiated by the addition of an NADPH generating system (163 μM ß-NADP+, 1.7 mM glucose-6-phosphate, and 0.375 U glucose-6-phosphate dehydrogenase). The incubation was conducted in a shaking water bath at 37°C. Aliquots (100 μl) were removed at 1, 3, 5, 10, 20, and 30 min, added to 100 μl of cold methanol and centrifuged at 13,500 x *g* for 1 min. An aliquot of the supernatant (20 μl) was then injected immediately into the HPLC for analysis. The concentration of MAQ-NOH in the microsomal incubations was quantified by measuring the peak height against a standard curve obtained by addition of synthetic MAQ-NOH to microsomal protein.

To obtain a mass spectrum of the microsomal metabolite, three microsomal suspensions (1 ml) were concentrated using Oasis HLB 1 cc C_{18} Extraction Cartridges (Waters Assoc.,

Milford, MA). The cartridges were washed with 1 ml of methanol and equilibrated with 1 ml of water. After loading the microsomal incubation on the cartridge, it was washed with 5% methanol/water. The metabolite was eluted with 1 ml of 100% methanol and dried in a Speed Vac Concentrator. The three samples were then pooled and dissolved in 75% methanol/water, and injected onto the HPLC. The peak corresponding to the metabolite was collected, and an aliquot was injected directly into the ion source of the mass spectrometer for analysis.

Hemotoxicity Studies

The level of methemoglobin in erythrocyte suspensions treated with MAQ-NOH (100-2,500 μM) was measured as described previously (Harrison and Jollow, 1987). Briefly, 100 μl aliquots of the red cell suspensions were added to 5 ml of hemolysis buffer (0.277% potassium phosphate monobasic, 0.289% sodium phosphate dibasic, and 0.05% Triton X-100; pH7.8). The hemolyzed solution was divided into four 1 ml aliquots; 20 μl of 10% KCN was added to the second and forth aliquots, and 20 μl of 20% $KFe(CN)_6$ was added to the third and forth aliquots. The absorbance of each aliquot was determined at 635 nm. The percent methemoglobin of total hemoglobin was calculated as %methemoglobin = ((A1-A2)/(A3-A4))100.

The survival of ^{51}Cr-labeled red cells was determined *in vivo* after *in vitro* exposure to various concentrations of MAQ-NOH (0-750 μM) as described previously (Harrison and Jollow, 1986). Briefly, MAQ-NOH dissolved in DMSO (10 μl) was added to erythrocyte suspensions (40% red cells, 2.0 ml) and allowed to incubate aerobically for 2 hr at 37°C. After the incubation, the red cells were washed once, resuspended in PBSG, and 0.5 ml aliquots were administered intravenously to isologous rats. Thirty minutes after administration of the labeled red cells a T_0 blood sample was taken from the orbital sinus, and then serial samples were taken at 48 hr intervals for 14 days. At the end of the experiment, the radioactivity in the blood samples was counted concurrently in a well-type

gamma counter, and the counts above background were expressed as percentage of the T_0 sample. The time for blood radioactivity to decrease to 50% of initial levels ($^{51}CrT_{50}$) was determined for each animal by regression analysis. Statistical significance was determined with the use of Student's t-test.

To determine the hemolytic activity of MAQ-NOH *in vivo*, untreated ^{51}Cr-labeled red cells were administered intravenously to isologous rats. A T_0 blood sample was taken via the orbital sinus 48 hr after administration of the labeled cells. MAQ-NOH (150 and 250 mg/kg, i.p.) dissolved in DMSO (0.5 ml/kg, i.p.) was administered immediately after the T_0 blood sample was taken. Serial blood samples were taken at the designated intervals and assayed as described above for the *in vitro* incubation/*in vivo* survival protocol.

Results

Microsomal Metabolism of 6-MAQ

To determine whether an N-hydroxy metabolite of 6-MAQ could be formed by rat and human liver microsomes, 6-MAQ (1 mM) was incubated with liver microsomes (1 mg/ml) containing an NADPH-generating system for 3 min at 37°C. At the end of the incubation period, an aliquot (100 μl) was removed, added to ice-cold methanol (100 μl) and centrifuged. An aliquot of the supernatent (20 μl) was then injected immediately onto the HPLC-EC. As shown in fig. 2.5C, a single chromatographic peak was observed from the rat microsomal sample. This peak was not observed in the absence of an NADPH-generating system (fig. 2.5B). The retention time of the metabolite peak was identical to that of synthetic MAQ-NOH (fig. 2.5A), and its UV-visible absorbance spectrum also was identical (data not shown). Formation of the metabolite was time dependent, being linear for about 3-5 minutes (fig 2.6A).

To confirm the identity of the microsomal metabolite, rat microsomal incubations were analyzed by ESI mass spectrometry. To generate a sufficient amount of the metabolite for

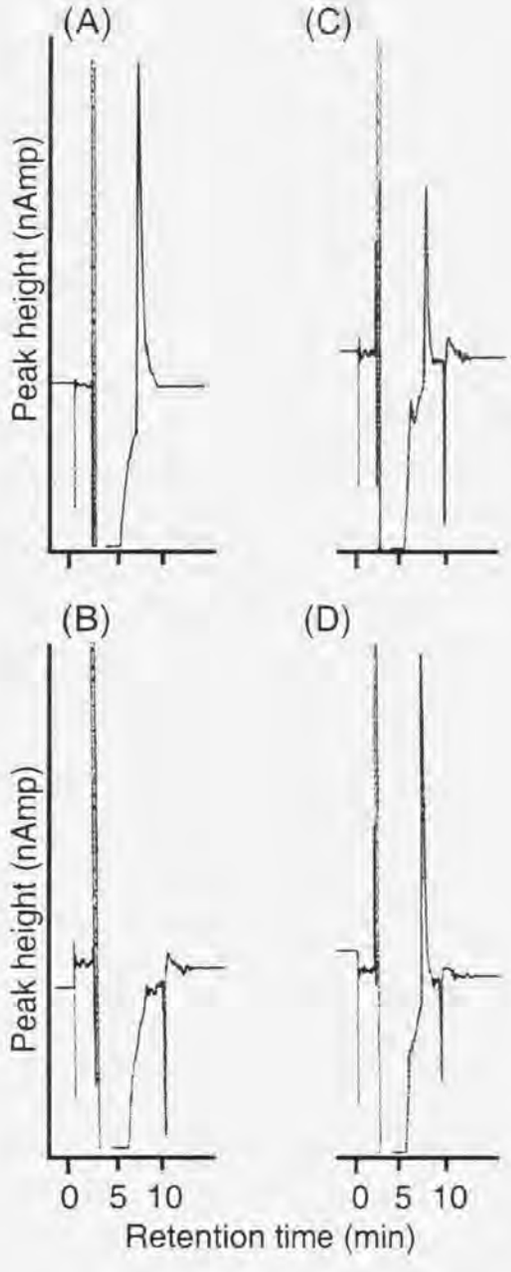

Fig. 2.5. HPLC-EC detection of MAQ-NOH. (A) Synthetic MAQ-NOH standard (1 μmol/ml); (B) 6-MAQ (1 mM) incubated for 3 min in rat liver microsomes without an NADPH-generating system; (C) 6-MAQ incubated for 3 min in rat liver microsomes with an NADPH-generating system; (D) 6-MAQ incubated for 3 min in human liver microsomes with an NADPH-generating system.

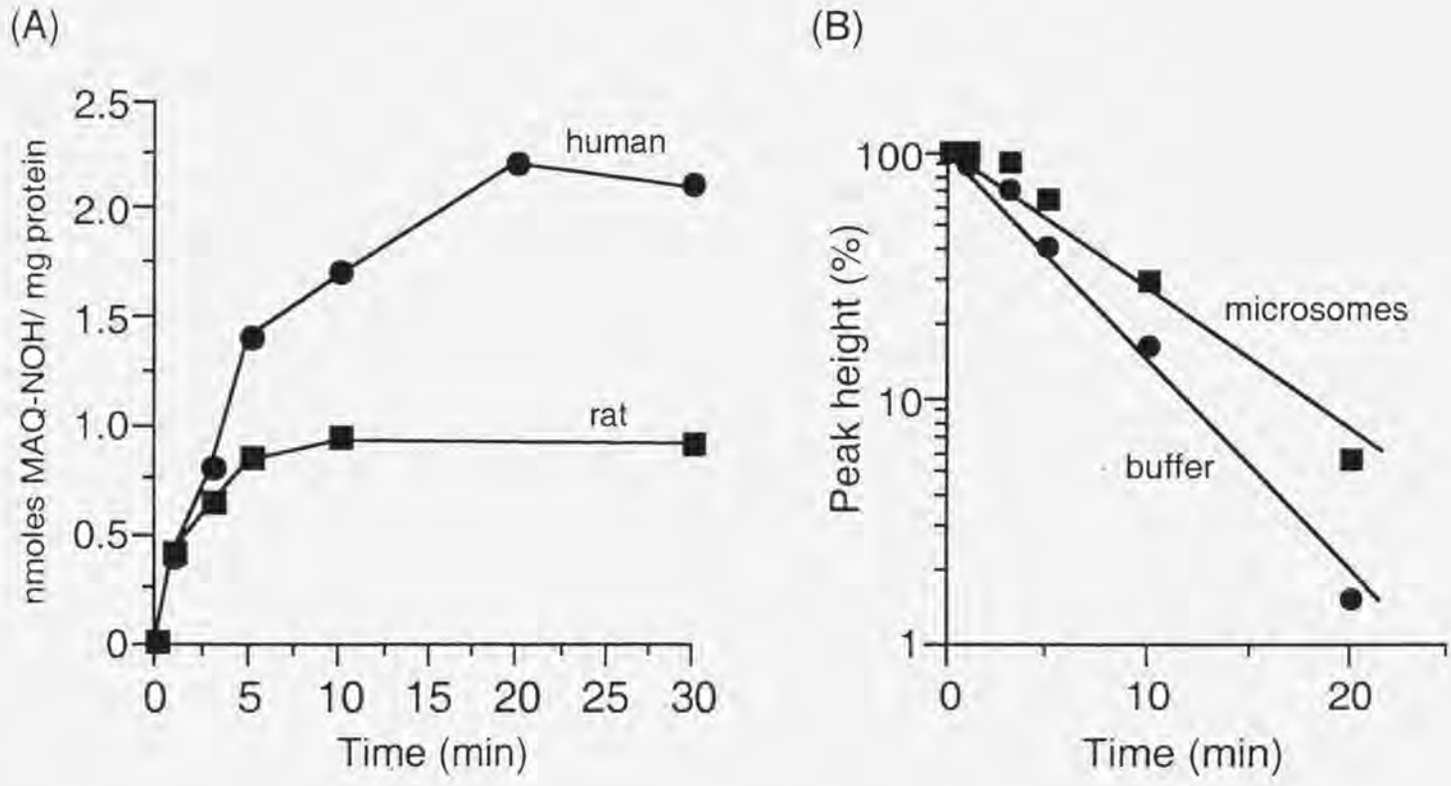

Fig. 2.6. (A) Time-dependence of MAQ-NOH formation in rat and human liver microsomes incubated with 6-MAQ (1 mM). Data points are means of duplicate incubations. (B) Stability of MAQ-NOH in buffer and microsomes. Synthetic MAQ-NOH was added to phosphate buffer (pH 7.4) or rat liver microsomes that did not contain an NADPH-generating system. Aliquots were withdrawn at designated intervals and the amount of MAQ-NOH remaining vs. time was quantified by HPLC-EC. Values are means of duplicate incubations.

MS analysis, three separate microsomal incubations were carried out with 6-MAQ (0.1 mM) for 10 min, and then the reaction mixtures were pooled and subjected to solid-phase extraction. The extract was concentrated in a Speed Vac, injected onto the HPLC, and the metabolite peak was collected and injected into the mass spectrometer. The mass spectrum of the metabolite (fig. 2.7A) showed a molecular ion at *m/z* 191, consistent with the addition of an hydroxyl group on 6-MAQ. A fragment ion at *m/z* 175 (M+–16, –O) was also observed, indicating the loss of elemental oxygen from the molecular ion. This loss of 16 *m/z* units has been observed previously with other N-hydroxyarylamine compounds (Lay et. al., 1986). The molecular ion (*m/z* 191) was then selected and subjected to ESI MS/MS. The MS/MS spectrum of the microsomal metabolite (fig. 2.7B) showed three fragment ions at *m/z* 176 (–15; $-CH_3$), 173 (–18; $-H_2O$), and 162 (–29; –CHO). This fragmentation pattern was also seen in the MS/MS of synthetic MAQ-NOH.

Incubation of 6-MAQ with human microsomes in the presence of NADPH (fig. 2.5D) resulted in the formation of a metabolite with the same retention time and UV spectrum as that of the rat (fig. 2.5C). The formation of MAQ-NOH by human microsomes was linear for about 5 minutes (fig. 2.6A). Of interest, the extent of formation of MAQ-NOH by human microsomes was about twice that of rat microsomes.

Parallel stability studies, in which a known amount of MAQ-NOH was added to rat microsomal suspensions, indicated that MAQ-NOH disappeared in an apparent first-order manner with a $T_{1/2}$ of about 6 minutes (fig. 2.6B). Thus it is likely that the observed production of MAQ-NOH in rat and human microsomal suspensions is an underestimation of the enzymatic capacity of the microsomes to N-oxidize 6-MAQ.

In vivo hemolytic activity of MAQ-NOH

To examine the hemolytic potential of MAQ-NOH *in vivo*, groups of rats were infused with ^{51}Cr-labeled erythrocytes 48 hr before i.p. administration of various doses of MAQ-NOH (dissolved in DMSO) or the vehicle alone. After an initial blood sample (T_0), serial

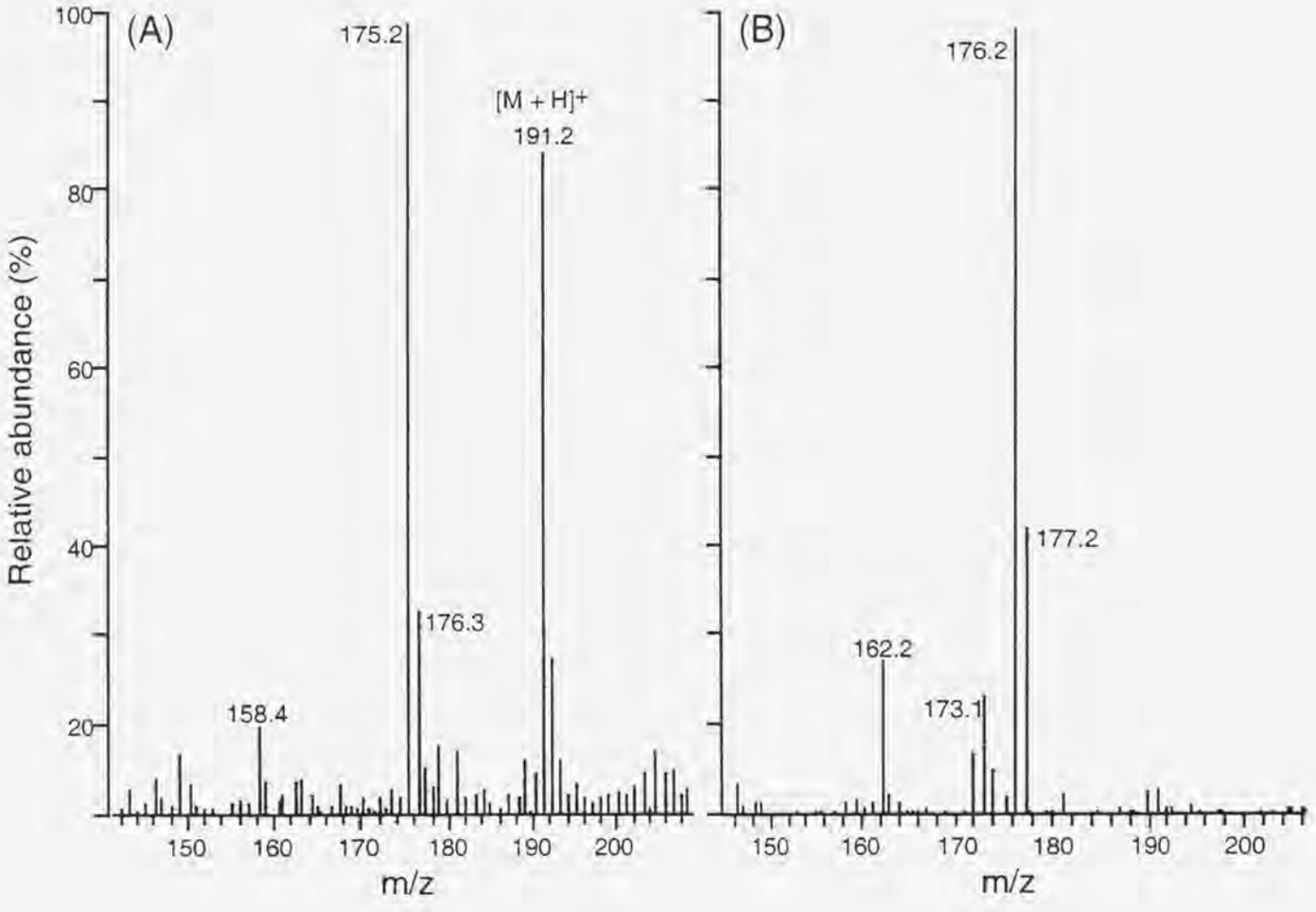

Fig. 2.7. (A) ESI mass spectrum of the 6-MAQ metabolite formed in rat liver microsomes. (B) ESI MS/MS of the parent ion (*m/z* 191).

blood samples were obtained to allow determination of the time necessary for the radioactivity to decline to 50% of the T_0 value (^{51}Cr-T_{50}) for each animal. As shown in fig. 2.8, vehicle-treated controls exhibited a gradual decline in blood radioactivity (^{51}Cr-T_{50} of 9.27 ± 0.34 days) that is considered to reflect primarily the normal removal of senescent erythrocytes. Erythrocyte survival after administration of a 150 mg/kg (0.94 mmol/kg) dose of MAQ-NOH was not significantly different from controls. However, the 250 mg/kg (1.57 mmol/kg) dose produced a statistically significant increase in the rate of removal of the radiolabeled erythrocytes (^{51}Cr-T_{50} of 6.36 ± 1.1 days). Unfortunately, administration of higher doses of MAQ-NOH (>300 mg/kg) were lethal due to acute respiratory depression, which precluded the construction of a complete dose-response curve for MAQ-NOH hemolytic activity *in vivo*.

Direct Hemotoxicity of MAQ-NOH

To determine if MAQ-NOH acts directly upon red cells to produce hemolytic damage, rat ^{51}Cr-labeled erythrocytes were resuspended in PBSG (40% hematocrit) and incubated with various concentrations of MAQ-NOH *in vitro* for 2 hr at 37°C. No evidence for hemolysis was observed under these incubation conditions. The cells were then washed once, administered intravenously to isologous rats, and serial blood samples were taken for 15 days. As shown in fig. 2.9A, exposure of the labeled cells to MAQ-NOH induced a concentration-dependent increase in the rate of removal of radioactivity from the blood as compared to controls. The concentration-dependence of this response is shown in fig. 2.9B; the hemolytic response is plotted as the percentage reduction in the ^{51}Cr-T_{50} of the experimental animals relative to the mean control value. The EC_{50} under these experimental conditions was about 350 μM. In contrast to MAQ-NOH, treatment of ^{51}Cr-labeled erythrocytes with 6-MAQ (1.5 mM) did not increase significantly the rate of removal of radioactivity from the blood as compared to controls (data not shown).

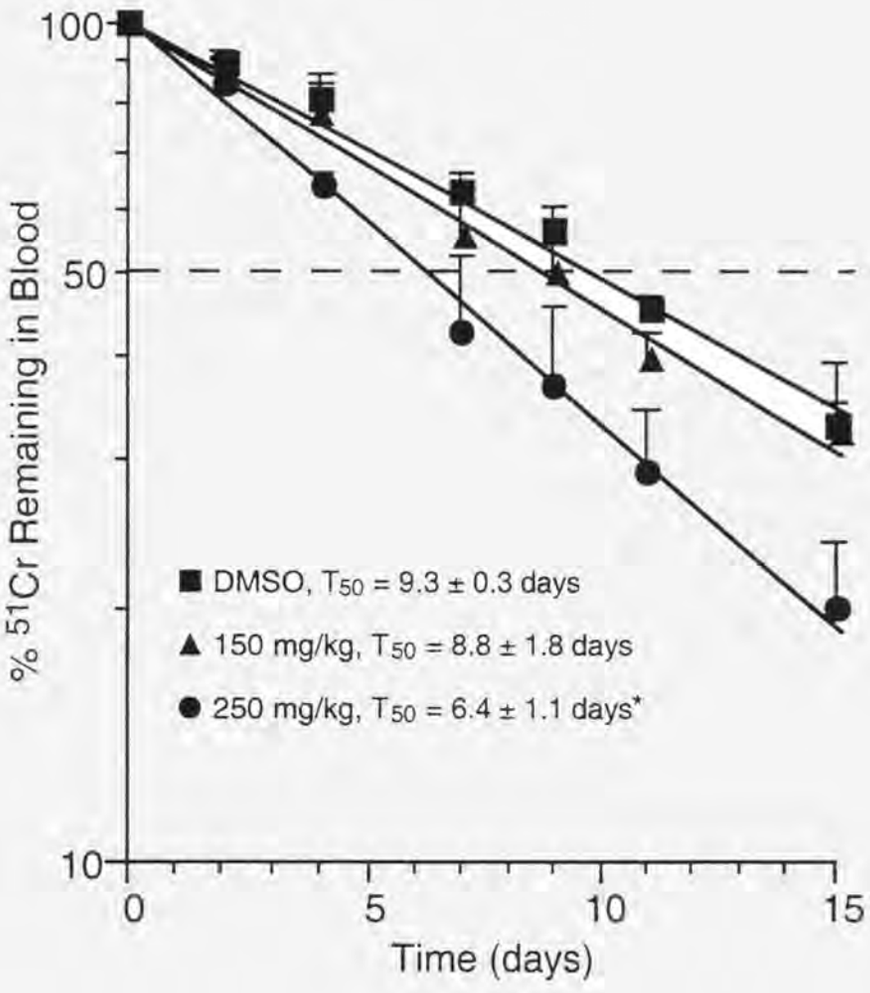

Fig. 2.8. Effect of administration of MAQ-NOH to rats on the survival of ^{51}Cr-labeled erythrocytes *in vivo*. Isologous rats received untreated radiolabeled erythrocytes intravenously 48 hr before i.p. administration of the indicated doses of MAQ-NOH dissolved in DMSO; control rats were injected with vehicle alone. T_0 blood samples were obtained from the orbital sinus immediately before MAQ-NOH administration. Data points are means ± SD (n=3); *$p \leq 0.05$.

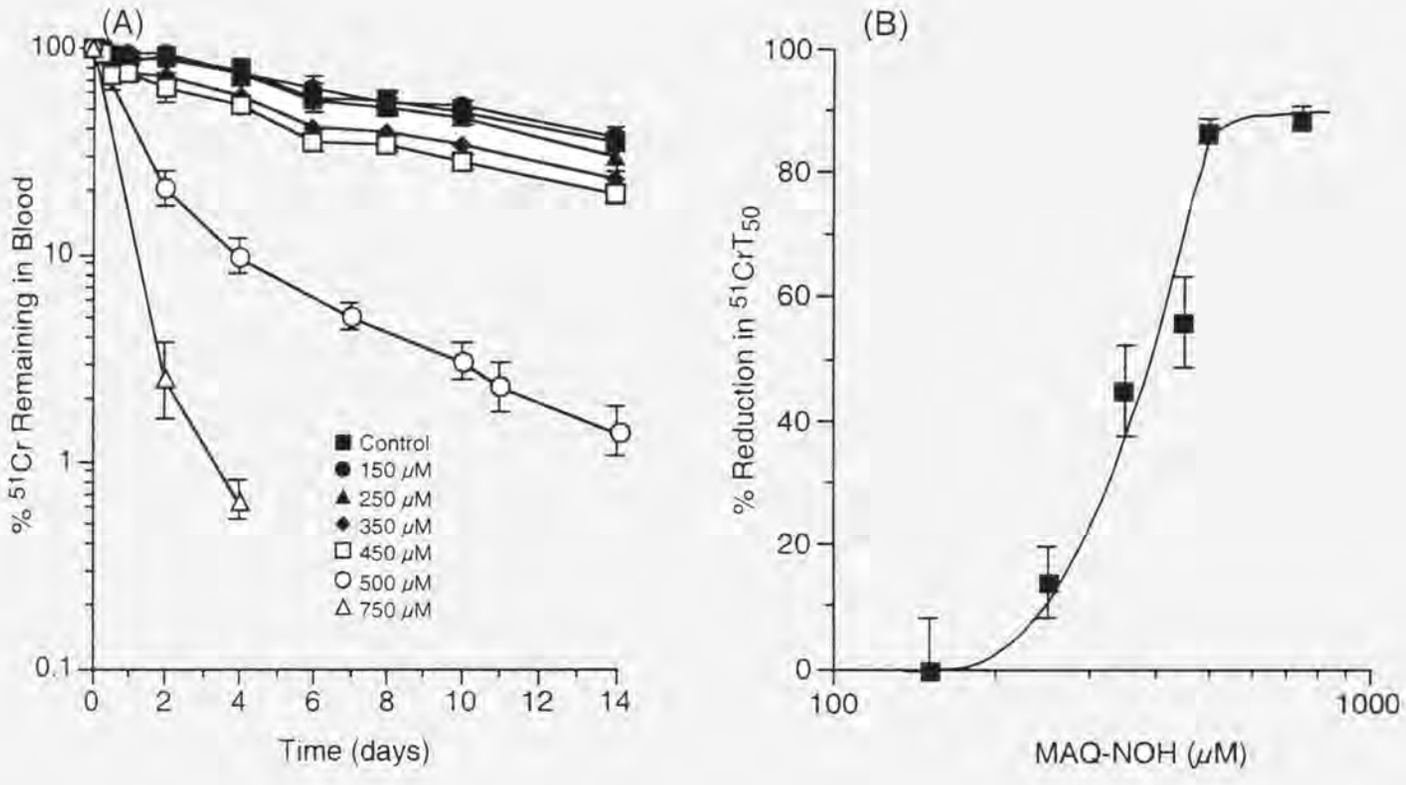

Fig. 2.9. (A) Survival of ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure of the labeled cells with MAQ-NOH. Radiolabeled erythrocytes were incubated for 2 hr at 37^{0}C with the indicated concentrations of MAQ-NOH; control cells were incubated with vehicle (10 μl DMSO) alone. The erythrocytes were then washed and administered intravenously to isologous rats. T_0 blood samples were taken 30 min after administration of the labeled cells. Data points are means ± SD (n=4). (B) Concentration-response relationship for reduction in the T_{50} of ^{51}Cr-labeled erythrocytes after MAQ-NOH exposure. The values are means ± SD (n=4).

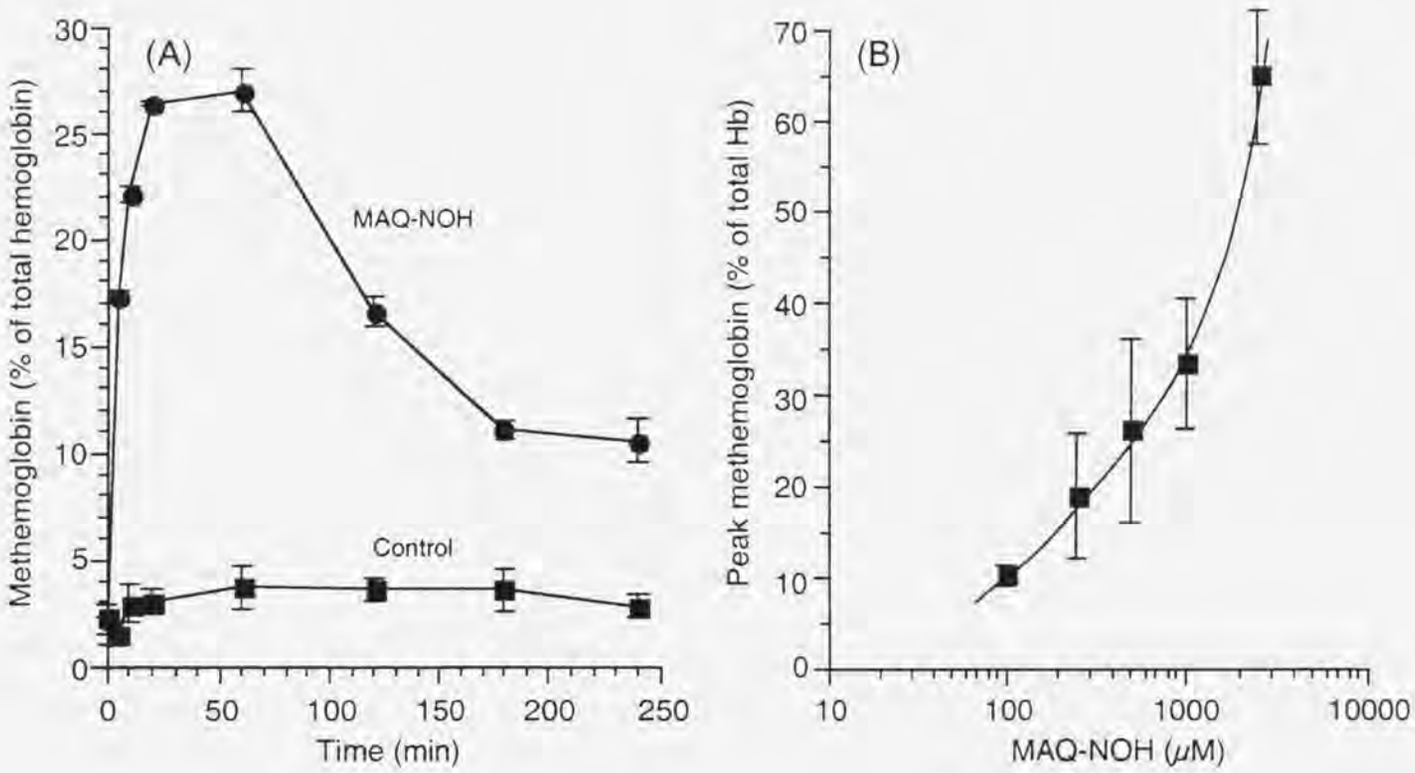

Fig. 2.10. (A) Methemoglobin formation *vs.* time in rat erythrocytes treated with the vehicle (DMSO, 10 μl) or MAQ-NOH (750 μM). Data points are means ± SD (n=4). (B) Concentration dependence of the methemoglobinemic response to MAQ-NOH. Peak methemoglobin, the maximal level of methemoglobin achieved for each incubation. Data points are means ± SD (n=4).

To examine the capacity of MAQ-NOH to induce methemoglobinemia *in vitro*, the time- and concentration-dependence of methemoglobin formation was examined in rat erythrocyte suspensions exposed to MAQ-NOH. As shown in fig. 2.10A, incubation of erythrocyte suspensions with MAQ-NOH (750 μM) resulted in the rapid formation of methemoglobin. Methemoglobin levels reached a peak of about 25% within an hour of MAQ-NOH exposure, and then declined gradually over the next 2 hrs. In contrast, the level of methemoglobin in control incubates remained low and constant during the course of the experiment. The MAQ-NOH concentration dependence of this response after 60 min of incubation is shown in fig. 2.7B. Methemoglobin levels under these conditions ranged from about 10% methemoglobin at 100 μM MAQ-NOH up to about 65% methemoglobin at 2.5 mM MAQ-NOH.

Discussion

The present results demonstrate that 6-MAQ, a known human metabolite of primaquine, can be N-hydroxylated to form MAQ-NOH by both rat and human liver microsomes (fig. 2.5C and D). Incubation of rat red cells with this metabolite caused a concentration-dependent formation of methemoglobin (fig. 2.10B). When ^{51}Cr-tagged red cells were incubated with MAQ-NOH, washed, and then administered to isologous rats, survival of the tagged red cells in the circulation was reduced in a concentration-dependent manner. Under the incubation conditions chosen (aerobic for 2 hr at 37°C in buffer containing glucose), no evidence for frank hemolysis was observed, and the EC_{50} for the hemolytic response was about 350 μM MAQ-NOH (fig. 2.9B). Furthermore, MAQ-NOH was able to provoke a hemolytic response when administered directly to rats (fig. 2.8).

Although the crucial role of metabolism in primaquine hemotoxicity has been accepted for over 40 years (Fraser and Vesell, 1968), the hemotoxic metabolite(s) have not been identified. Lack of progress is due in large part to the multiplicity of the known and proposed pathways of primaquine metabolism (fig. 1.2), all of which can give rise to

metabolites with redox potential. Moreover, poor organic solubility and instability of these putative metabolites have complicated their extraction and quantification from biological media (Idowu et al., 1995). These obstacles, combined with the difficulty in provoking a hemolytic response in laboratory animals with primaquine (Lee et al., 1981; Jollow et al., unpublished studies), have hampered efforts to determine the contribution each metabolite makes towards methemoglobin formation and hemolytic damage *in vivo*.

To overcome these problems, investigators have synthesized phenolic derivatives of primaquine and have examined their effects in erythrocyte suspensions. Following the suggestion of Tarlov and colleagues (Tarlov et al., 1962), much attention has been given to the 5-hydroxy- and 5,6-dihydroxy metabolites of primaquine (fig. 1.2), which (via 5,6-quinone formation) could support redox cycling and the generation of active oxygen species (Link et al., 1985). In support of this postulate, these compounds were shown to induce a variety of oxidative effects in both normal and G6PD-deficient red cells, including stimulation of hexose monophosphate shunt activity (Baird et al., 1986), hemoglobin oxidation and GSH depletion (Strother et al., 1981; Agarwal et al., 1988; Fletcher et al., 1988). More recently, Vasquez-Vivar and Augusto (1994) showed that a 500 μM concentration of the quinoneimine derivatives of these metabolites (prepared by treatment of the phenols with hydrogen peroxide) could induce oxidative damage and increase the osmotic fragility of rat erythrocytes. When examined under the same experimental conditions, 6-MAQ was found by these investigators to be inactive. Thus, they concluded that phenolic metabolites were the toxic species and that an N-hydroxylated metabolite was unlikely to contribute to the hemotoxicity of primaquine. However, MAQ-NOH was not synthesized in these studies and tested directly, and it is not clear that sufficient levels of MAQ-NOH could have been generated from 6-MAQ under their experimental conditions to allow adequate test of the postulated role of this metabolite. Moreover, since hemolytic anemia in humans is associated with sequestration of intact red cells rather than with

intravascular hemolysis (Rifkind, 1966), the relevance of their observed increases in osmotic fragility is uncertain.

While the *in vivo* survival data in fig. 2.9 clearly indicates that MAQ-NOH is directly hemolytic to G6PD-normal rat red cells, these data do not allow for direct assessment of the role of MAQ-NOH in primaquine hemotoxicity. It is noteworthy, however, that the pattern of the response produced by MAQ-NOH exposure; i.e., the rate of ^{51}Cr uptake with no evidence of frank lysis, was similar to that observed with arylhydroxylamine metabolites of aniline and dapsone. The type of damage inflicted by these direct-acting hemolytic agents has been shown to be consistent with an acceleration of the normal removal of "aged" but intact red cells from the circulation by the spleen (Jollow and McMillan, 1998). The hemolytic potency of MAQ-NOH (EC_{50} 350 μM) towards G6PD-normal rat red cells, as measured in the *in vitro/in vivo* assay, was about 2-fold lower than that of dapsone hydroxylamine (EC_{50} ca. 150 μM) which mediates the hemolytic activity of dapsone (Grossman and Jollow, 1988), and about three-fold more potent than N-hydroxyphenetidine (EC_{50} ca. 900 μM), which mediates the hemotoxicity of phenacetin (Jensen and Jollow, 1991).

The *in vitro* exposure/*in vivo* erythrocyte survival assay allows the hemolytic damage observed *in vivo* to be reproduced *in vitro* under controlled conditions during a 2-hr incubation period before the cells are returned to the circulation of isologous rats, and thus is a useful indicator of relative hemolytic potency among direct-acting hemolytic agents. However, this assay cannot be utilized to define the MAQ-NOH blood concentrations needed to cause a hemolytic response *in vivo*. Previous studies with dapsone hydroxylamine have demonstrated that hemolytic activity is proportional to the AUC of the metabolite and is not dependent on its concentration (Grossman and Jollow, 1988). Since the half-life of primaquine in humans is about 6 hours (Fletcher et al., 1981), it is likely that 6-MAQ and the secondary metabolite, MAQ-NOH, will be produced at a low but steady rate during chronic primaquine therapy. Thus, while the concentration of MAQ-NOH in the circulation

may never be very high, its AUC may be appreciable and reach toxicologically significant levels in G6PD-deficient patients.

Assessment of the contribution that MAQ-NOH makes to primaquine hemotoxicity is further complicated by the probability that the putative phenolic metabolites discussed above are formed in humans and hence also have the potential to induce hemolytic injury. In preliminary studies using the *in vitro* exposure/*in vivo* erythrocyte survival assay, we have observed that both 5-hydroxyprimaquine and 5-hydroxy-6-desmethylprimaquine are direct-acting hemolytic agents with hemolytic potencies similar to that of MAQ-NOH (unpublished results). Thus, primaquine could be metabolized to three types of hemotoxic species: phenolic, diphenolic and arylhydroxylamine, each of which would be capable of undergoing redox cycling within the red cell. 5-Hydroxyprimaquine forms a redox pair with its *p*-quinoneimine analog, the 5,6-diphenolic metabolite with either an *o*-quinone or a *p*-quinoneimine, and the 8-hydroxylamino metabolite (i.e., MAQ-NOH) with its nitroso analog. These considerations emphasize the need for more detailed studies on the metabolism of primaquine in rats and humans, and raise the possibility that more than one type of metabolite is responsible for primaquine hemotoxicity.

In the present studies on the metabolism of 6-MAQ, we did not detect a phenolic metabolite in either rat or human microsomal incubations. This does not indicate that these metabolites are not formed, but does raise the possibility that either they may not be major contributors to the hemotoxicity of 6-MAQ, or that their instability is much greater than that of MAQ-NOH under these experimental conditions. Further experiments will be necessary to determine whether phenolic metabolites of primaquine can be detected in humans. In addition, the effect of CYP inducers and inhibitors on the metabolism of primaquine and 6-MAQ will be necessary to identify CYP isoforms responsible for toxic *vs.* non-toxic pathways, and will be crucial in the development of a primaquine-sensitive animal model.

In regard to the mechanism underlying primaquine-induced hemolytic anemia, data published by Degowin et al. (1966) first raised the possibility that oxidative damage to

erythrocytes could arise by different mechanisms depending on the oxidant. These investigators reported that the doses of primaquine necessary to evoke signs of hemolysis in A$^-$ G6PD-deficient volunteers are about 20-fold lower than those required to elicit a similar response in G6PD-normal volunteers, whereas the doses of dapsone required to induce similar responses in G6PD-deficient *vs.* normal differ only by a factor of two. Although the underlying basis for these differences is not clear, it may be explained by the fact that dapsone hemotoxicity is mediated solely by N-hydroxy metabolites, whereas primaquine hemotoxicity could be mediated by multiple hemotoxic species, quinone, quinoneimine, and arylhydroxylamine (fig. 1.2). Clearly, a quantitative assessment of the contribution each type of metabolite makes towards the hemotoxic response will be important in elucidating the mechanism underlying primaquine-induced hemolytic anemia.

In summary, we have demonstrated that a known human metabolite of primaquine, 6-MAQ, can be N-hydroxylated by both rat and human liver microsomes, and that this N-hydroxy metabolite is hemolytic *in vivo* in rats, and is directly hemotoxic to the rat red blood cell. The contribution of this metabolite to primaquine hemotoxicity remains to be assessed.

CHAPTER 3

Primaquine-Induced Hemolytic Anemia: Effect 6-Methoxy-8-Hydroxylaminoquinoline on Rat Erythrocyte Sulfhydryl Status, Membrane Lipids, Cytoskeletal Proteins and Morphology

Introduction

The antimalarial drug primaquine has been the drug of choice for the treatment of the exoerythrocytic forms of *Plasmodium vivax* and *P. ovale* for more than 40 years (Tracy and Webster, 1996). Recently, primaquine has also been utilized for its gametocytocidal activity against *P. falciparium* to help combat widespread drug resistance of this species to blood schizonticides, such as chloroquine (Peters, 1999). Furthermore, primaquine is used to treat mild to moderate cases of *Pneumocystis carinni* pneumonia in patients with acquired immunodeficiency syndrome (Toma et al., 1998). Despite the clinical importance of primaquine, its therapeutic use is limited by its toxic side-effects, hemolytic anemia and methemoglobinemia (Dern et al., 1955; Degowin et al., 1966).

The mechanism by which primaquine induces hemolytic anemia remains elusive. Early mechanistic studies established that: 1) metabolite(s) of primaquine are the toxic species; 2) GSH is lost from the red cell prior to a hemolytic response; 3) denatured hemoglobin aggregates (i.e., Heinz bodies) are associated with the red cell membrane; and 4) hemolytic anemia is particularly pronounced in individuals who are deficient in glucose-6-phosphate dehydrogenase (G6PD) activity (for review, see Beutler, 1969). Collectively, these observations led to the concept that primaquine-induced hemolytic anemia is caused by oxidative stress. However, the identity of the toxic primaquine metabolites and the nature of the oxidant stress are not known.

We have reported recently that the known human primaquine metabolite, 6-methoxy-8-aminoquinoline (6-MAQ), can be N-hydroxylated to form 6-methoxy-8-hydroxylaminoquinoline (MAQ-NOH) by both rat and human microsomes (Bolchoz et al., 2001). Furthermore, MAQ-NOH was found to be a direct-acting hemolytic agent in the rat; that is, when rat ^{51}Cr-labeled red cells are incubated with MAQ-NOH *in vitro* and returned to isologous rats, the labeled red cells are more rapidly removed from the circulation than are the vehicle-treated control cells.

Although sequestration of senescent or damaged (but intact) red cells from the circulation is known to occur by macrophages of the reticuloendothelial system (Rifkind, 1966), the signal that marks these cells for removal remains unknown and continues to be a subject for debate (for review, see Bratosin et al., 1998). One group of studies suggests that peroxidative damage to the plasma membrane lipid bilayer transmits the signal for sequestration (Lubin and Chiu, 1982; Zwaal and Schroit, 1997). Alternatively, membrane skeletal protein alterations may underlie the removal process. For example, alteration in the lateral distribution of band 3 protein via hemoglobin (or hemichrome) binding has been shown to result in the binding of autologous antibodies, which commit the cells for uptake by cultured monocytes (Waugh et al., 1987; Turrini et al., 1991).

In view of the critical role proposed for oxidant damage in the mechanism underlying primaquine-induced hemolytic anemia, we have examined the effect of MAQ-NOH on sulfhydryl status, membrane lipids and skeletal proteins in suspensions of rat erythrocytes using MAQ-NOH concentrations known to induce the premature removal of these cells from the circulation. We report that MAQ-NOH has only a modest capacity to oxidize red cell GSH to GSSG and GS-protein mixed disulfides, and to induce the formation of

hemoglobin-skeletal protein adducts. In contrast to other arylhydroxylamines, exposure of normal rat red cells to MAQ-NOH significantly enhanced the peroxidation of membrane lipids. When GSH was depleted from rat red cells to mimic the low levels of GSH that are observed in the human G6PD-deficient red cell (Gaetani et al., 1979), the hemolytic activity of MAQ-NOH was markedly enhanced. This exacerbation was associated with the development of protein thiol oxidation without change in the level of lipid peroxidation. It is suggested that MAQ-NOH can inflict hemolytic injury on the red cell by two pathways; lipid peroxidation in GSH-normal red cells and protein oxidation in GSH-depleted red cells. The possibility of synergistic interaction between these processes and among the hemotoxic metabolites of primaquine is discussed.

Materials and Methods

Chemicals and Materials

MAQ-NOH was synthesized as described previously (Allahyari et al., 1984). Malondialdehyde, diethyl maleate (DEM), and rabbit anti-rat hemoglobin IgG were purchased from Sigma Chemical Co. (St Louis, MO). HRP-conjugated donkey anti-rabbit IgG was purchased from Amersham Pharmacia (Piscataway, NJ). $Na_2{}^{51}CrO_4$ in sterile saline (1 mCi/ml, pH 8) was obtained from New England Nuclear (Billerica, MA). All other chemicals were of the best commercially available grade.

Animals

Male Sprague-Dawley rats (75-100 g) were purchased from Harlan Laboratories (Indianapolis, IN) and were maintained on food and water *ad libitum.* Animals were acclimated to a 12-hr light-dark cycle prior to their use.

Red Cell Incubation Conditions

Blood from the descending aorta of anesthetized rats was collected into heparinized tubes and washed in isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM D-glucose (PBSG). After removal of the plasma, the red cells were resuspended in PBSG to a 40% hematocrit and used the same day they were collected. Experiments were carried out by addition of various concentrations of MAQ-NOH dissolved in DMSO (10 μl) to the erythrocyte suspensions (2 ml) and allowed to incubate at 37°C for up to 2 hr.

Determination of Sulfhydryl Status

GSH content was determined as described previously (Grossman et al., 1992). Aliquots (0.2 ml) of the incubation mixtures were lysed with 800 μl of cold EDTA (1 mM) and the protein precipitated with 500 μl of 25% sulfosalicylic acid. Following a 5 min incubation on ice, the protein was pelleted at 10,000 rpm for 2 min. To assay for GSH, 500 μl of the supernatant was added to 1 ml of Tris–acetate buffer (0.5 M; pH 7.6) and 2 ml of phosphoric acid (1 M). GSH content was analyzed by HPLC with electrochemical detection as described below.

To determine GSSG, 500 μl of supernatant was added to 1 ml of Tris–acetate buffer (0.5 M; pH 7.6) and 10 μl of n-octanol. The mixture was incubated with sodium

borohydride (50 mg) at 37°C for 30 min. After incubation, the mixture was chilled, 2 ml of phosphoric acid (1M) was added, and GSH content analyzed.

To determine GS-protein mixed-disulfides the protein pellet was washed with 25% sulfosalicylic acid, suspended in a solution containing 2.970 ml of 25% sulfosalicylic acid, 15 μl Triton X-100, and 15 μl Antifoam, homogenized for 30 sec in a Polytron homogenizer (Brinkman), and centrifuged at 15,000 rpm for 10 min. The pellet was treated with sodium borohydride (50 mg), homogenized for 30 sec, and incubated at 37°C for 30 min. After incubation, the mixture was chilled, treated with 2 ml of phosphoric acid (1M), centrifuged at 15,000 rpm for 10 min, and the content of solubilized GSH determined.

GSH content was determined by HPLC with electrochemical detection. Chromatography was performed on a Waters HPLC system (Waters Assoc., Milford, MA) consisting of a Model 6000A pump, a Rheodyne injector (10 μl loop) and a 3.9 x 150 mm Waters Nova Pak C8 column. GSH was eluted with 8% methanol in 50 mM potassium phosphate buffer (pH 2.4) containing 5 mM heptanesulfonic acid with a flow rate of 1.2 ml/min and detected on a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a Au/Hg amalgam electrode operating at +100 V. The amount of sulfhydryl present in the samples was determined by comparison of peak heights to standards prepared identically to the samples.

Morphological Examination of Red Cells

After incubation, red cells treated with the vehicle (DMSO) or MAQ-NOH were washed and prepared for scanning electron microscopy as previously described

(Grossman et al., 1992). The red cells (1 ml aliquots) were fixed in 0.1 M cacodylate buffer containing 1% glutaraldehyde and 2% formalin for 20 min. The cells were affixed onto 0.2-μm filters using a Swinney connector attached to a syringe. The cells were fixed an additional 40 min as above, washed in saline, and dehydrated in a graded series of ethanol solutions ranging from 50-100% ethanol. The cells were then dried by critical point drying, cast with a thin coat of carbon and gold, and examined in a JEOL JSM-5410LV scanning electron microscope operating at 10 kV accelerating voltage and no tilt.

Preperation of Red Cell Ghosts

Red cell ghosts were prepared from vehicle- and MAQ-NOH-treated red cells as described previously (Grossman et al., 1992). After incubation, washed red cells were lysed in 20 ml of phosphate buffer (5 mM, pH 8.0) containing 20 mg of phenylmethylsulfonylfluride and centrifuged at 13,500 rpm for 10 min. The supernatant was removed by aspiration. The ghosts were washed extensively in phosphate buffer (3-4 washes) to remove any unbound hemoglobin. Red cell ghosts were also prepared from red cells treated with dapsone hydroxylamine (DDS-NOH), and were used as a positive control for the presence of hemoglobin-skeletal protein adducts (Grossman et al., 1992).

Determination of Lipid Peroxidation in Red Cells

Red cells were analyzed for thiobarbituric acid-reactive substances (TBARS) as previously described (McMillan et al., 1998). Following a 60 min incubation in the presence of MAQ-NOH, aliquots (900 μl) of the suspension were added to 76.5 μl of

trichloroacetic acid to precipitate the proteins and centrifuged at 13,000 x g for 2 min. The supernatants (600 μl) were incubated with thiobarbituric acid (600 μl 0.67% in water) at 100°C for 15 min. After the samples cooled to room temperature, their absorbance was read at 532 nm. TBARS was quantitated based on a standard curve generated with known amounts of malondialdehyde prepared identically to the samples. Statistical significance was determined with the use of the Student's T-test.

Lipid peroxidation was also assessed by measuring the level of F_2-isoprostanes in red cell ghosts prepared from MAQ-NOH-treated red cells as described previously (Morrow and Roberts, 1999). Membrane phospholipids were extracted from the red cell ghosts and subjected to alkaline hydrolysis to release esterified F_2-isoprostanes. Free F_2-isoprostanes were converted to pentafluorobenzyl ester trimethylsilylether derivatives and measured by GC/MS. F_2-isoprostanes were quantitated based on a standard curve generated with known amounts of the F_2-isoprostane [$^2H_4$]8-iso-$PGF_{2\alpha}$.

Electrophoretic Analysis of Membrane Skeletal Proteins

Red cell ghosts (100 μl) were solubilized in 40.6 μl solubilization buffer (7% SDS, 35% glycerol, 25 mM EDTA, 0.28 M Tris-base). Aliquots (containing 35 μg of protein) were resolved on non-reducing, continuous gels consisting of 5% monomer and 1.5% bis-acrylamide crosslinker as described by McMillan *et. al.*. (McMillan et al., 1995). The resolved proteins were either stained with Gel Code Blue (Pierce, Rockford, IL) or transferred to PVDF membranes according to the method of Towbin *et. al.* (Towbin et al., 1979). The skeletal proteins were resolved Protein bands were identified according to their migration distance (Fairbanks et al., 1971).

Blotted proteins were blocked in TBST (Tris-buffered saline containing 0.5% Tween-20, pH 7.5) containing 5% w/v non-fat dry milk at 27°C overnight, washed 3 times in TBST and incubated in TBST containing 0.1% bovin serum albumin (fraction V) and primary antibody (diluted 1:10,000) at room temperature for 1 hr. The blotted proteins were washed 3 times in TBST containing 5% w/v non-fat dry milk and incubated with the peroxidase-conjugated secondary antibody at room temperature for 1 hr. Blots were developed with ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

GSH Depletion of Red Cell Suspensions

DEM dissolved in acetone was added to packed red cells at an initial concentration of 0.121 μl/ml packed cells. After a 15 min incubation at 37°C the red cells were analyzed for GSH content by HPLC with electrochemical detection as described above. This treatment typically reduced GSH concentrations to about 5-10% of the initial level, and this level of depletion was maintained throughout the course of the experiment. The cells were then resuspended in PBSG (40% suspension) and used on the same day that they were collected.

Determination of the Hemolytic Response

The survival of ^{51}Cr-labeled red cells *in vivo* after *in vitro* exposure to MAQ-NOH was determined as described previously (Harrison and Jollow, 1986). After the incubation, the red cells were washed, resuspended (40% hematocrit), and an aliquot (0.5 ml) was administered i.v. to isologous rats. T_0 blood samples were taken from the orbital sinus 30 min after the administration of the labeled cells. Additional samples were taken

in 48 hr intervals for 14 days. At the end of the experiment the samples were counted in a well-type gamma counter, and the data were expressed as a percentage of the T_0 blood sample. Each data point represents four rats.

Results

Effect of MAQ-NOH on Rat Erythrocyte Sulfhydryl Status

It has been shown that GSH levels are decreased in G6PD-deficient individuals given primaquine (Tarlov et al., 1962; Beutler, 1969). Thus, the effect MAQ-NOH on GSH levels was examined in rat erythrocytes *in vitro* under previously established hemolytic conditions (Bolchoz et al., 2001). MAQ-NOH was added to rat red cell suspensions, and aliquots were taken at various time points and analyzed for GSH, GSSG and GS-protein mixed disulfides by HPLC. As shown in fig. 3.1A, addition of a TC_{50} concentration of MAQ-NOH (350 μM) to rat red cells resulted in a transient decline of GSH to about 70% of initial levels, reaching a nadir within 30 min. The loss of GSH was matched by an increase in both GSSG and GS-protein mixed disulfides.

Examination of the concentration dependence of the effect of MAQ-NOH on erythrocyte GSH levels indicated that the pattern of the response was unchanged over the range of 350-1000 μM. When the nadir of GSH content (at 30 min) was plotted against MAQ-NOH concentration (fig. 3.1B), the EC_{50} of GSH depletion by MAQ-NOH was estimated to be about 1000 μM. Of interest, this concentration-response curve for MAQ-NOH-induced GSH oxidation is shifted significantly to the right of the concentration-

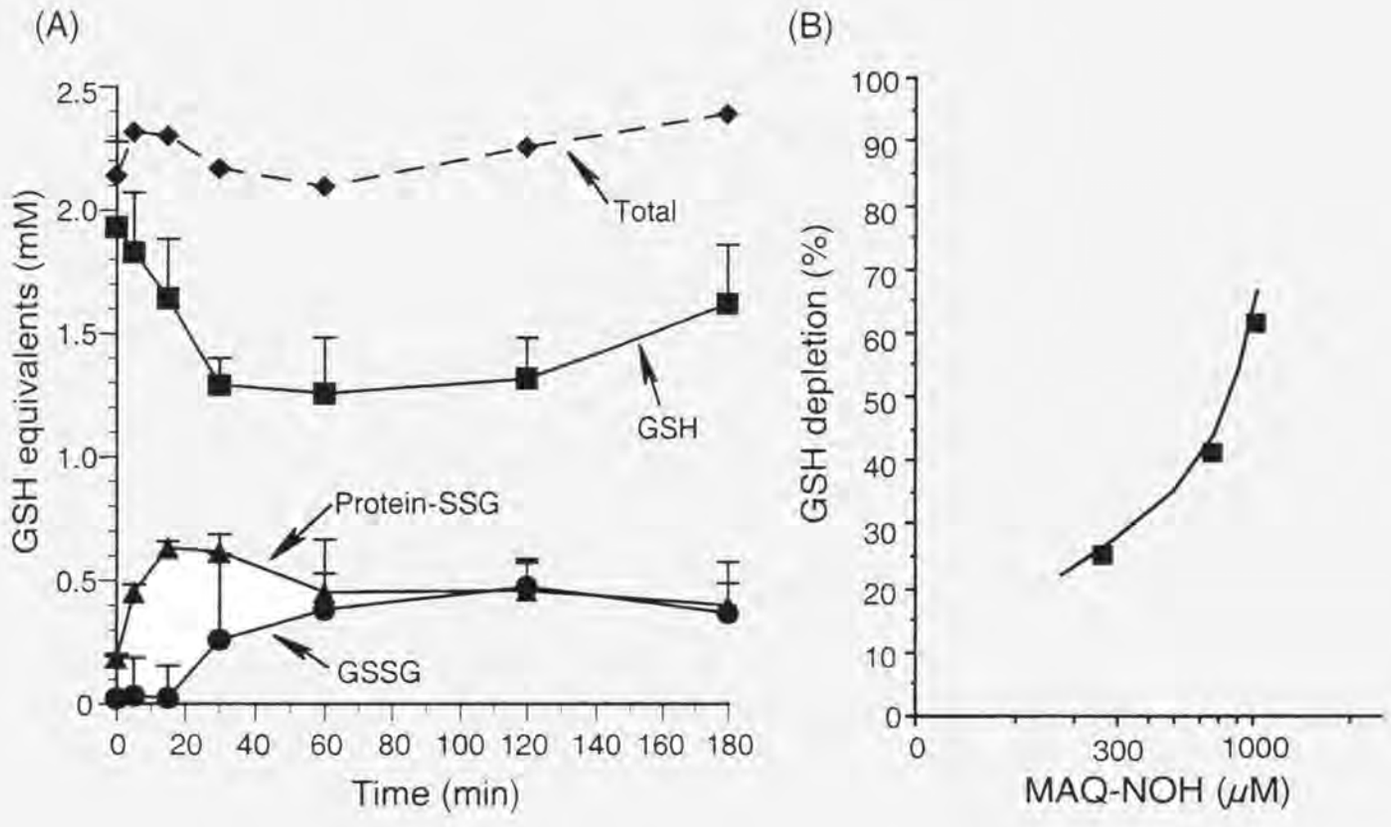

Fig. 3.1. (A) Effect of MAQ-NOH on rat erythrocyte sulfhydryl status. Rat erythrocytes were incubated at 37°C in PBSG containing MAQ-NOH (350 μM). At the indicated time points aliquots were withdrawn and assayed for GSH, GSSG, and GS-protein mixed disulfides. The values shown are means $\pm$ SD (n=3). (B) Concentration response relationship for GSH oxidation by MAQ-NOH in rat erythrocytes. Percent reduction in GSH was determined using the nadir values (30 min) for GSH as compared to controls. Values are means of duplicate determinations.

response curve previously reported for MAQ-NOH hemolytic activity (TC_{50} ca. 350 μM) (Bolchoz et al., 2001).

Effect of MAQ-NOH on Rat Erythrocyte Membrane Lipids

To determine whether lipid peroxidation could be detected in rat red cell suspensions exposed to MAQ-NOH, TBARS formation was measured after a 60 min incubation with MAQ-NOH. As shown in fig. 3.2A, the amount of TBARS formed in MAQ-NOH-treated red cells was increased significantly as compared to the vehicle-treated control cells. TBARS formation was dependent on MAQ-NOH concentration up to 350 μM.

Although TBARS is a widely accepted method for the measurement of lipid peroxidation, problems with the specificity of this assay are well known (Moore and Roberts, 1998). Thus, F_2-isoprostane content in MAQ-NOH-treated red cell ghosts was used to confirm the induction of lipid peroxidation. Following a 2-hr incubation with various concentrations of MAQ-NOH, the cells were washed and lysed in hypotonic saline to prepare red cell ghosts. Esterified isoprostanes were hydrolyzed and extracted, and the content of free F_2-isoprostanes in the sample was quantified by GC/MS. As shown in fig. 3.2B, MAQ-NOH induced a concentration-dependent increase in the formation of F_2-isoprostanes, which confirmed the results obtained with the TBARS assay.

Effect of MAQ-NOH on Rat Erythrocyte Membrane Skeletal Proteins

Membrane skeletal proteins from control, DDS-NOH (included as a positive control) and MAQ-NOH-treated red cells were separated on SDS-PAGE gels and either stained

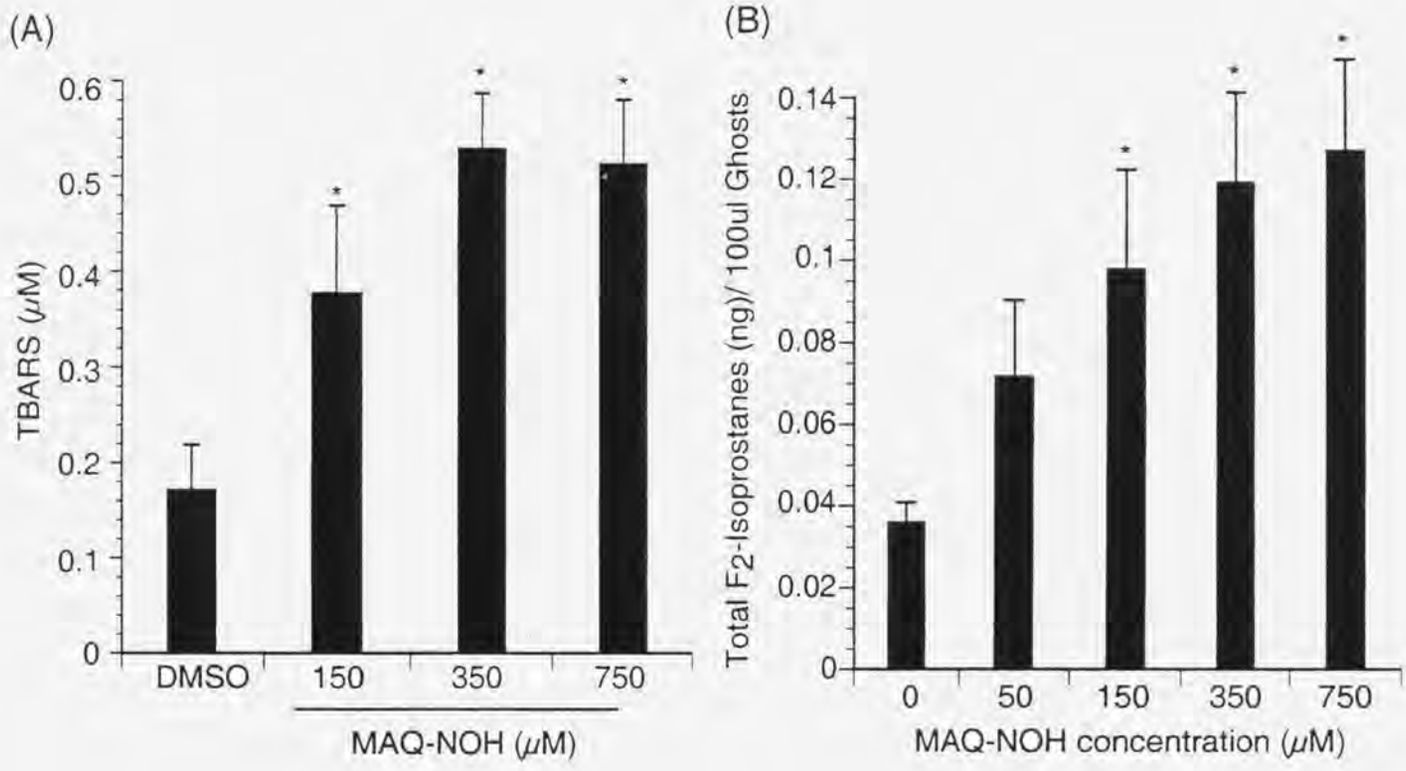

Fig. 3.2. (A) Formation of TBARS in rat erythrocytes exposed *in vitro* to MAQ-NOH. Rat erythrocytes were incubated at 37°C for 60 min in PBSG containing the indicated concentrations of MAQ-NOH. Control cells were incubated in PBSG containing the vehicle (10 μl DMSO). After the incubation, the cells were lysed, centrifuged and analyzed for TBARS as described in "Materials and Methods". The values are means ± SD ($p < 0.05$, n=5). (B) Formation of F_2-isoprostanes in ghosts prepared from rat erythrocytes exposed *in vitro* to MAQ-NOH. Erythrocyte suspensions (40% HCT) were incubated with the indicated concentrations of MAQ-NOH for 2 hrs at 37°C. After incubation, erythrocyte ghosts were prepared and analyzed for F_2-isoprostane content as described in "Materials and Methods". The values are means ± SD (n=4). *Significantly different from control ($p < 0.05$).

with Gel Code Blue or transferred to PVDF membranes and immunostained with anti-hemoglobin polyclonal antibodies. As shown in fig. 3.3A, the electrophoretic pattern of membrane skeletal proteins of the positive control (DDS-NOH, lanes 6-7) showed the characteristic changes previously described (Grossman et al., 1992); *viz.*, broadening of protein bands 1 and 2, loss of bands 2.1 and 4.2, splitting of band 3, and the formation of hemoglobin aggregates. Immunoblot analysis using the anti-hemoglobin antibody (fig. 3.3B) confirmed that the changes in electrophoretic mobility were due to formation of hemoglobin adducts with the skeletal proteins, and of the presence of monomeric and polymeric forms of hemoglobin.

MAQ-NOH-treated red cells (fig. 3.3A, lanes 2-5) were not significantly different from the control (lane 1) in regard to the mobility of the skeletal proteins. The absence of significant levels of skeletal protein-hemoglobin adducts was confirmed by the anti-hemoglobin immunoblot (fig. 3.3B, lanes 2-5). Of interest, a concentration-dependent increase in the amount of hemoglobin monomer (16 kDa) was observed in the Gel Code Blue-stained gel, as well as in the anti-hemoglobin immunoblot (fig. 3.3B, lanes 2-5).

Effect of MAQ-NOH on Rat Erythrocyte Morphology

To investigate the effect of MAQ-NOH on rat erythrocyte morphology, red cell suspensions were incubated in the presence and absence of MAQ-NOH, and aliquots of the cells were prepared for scanning electron microscopy. As shown in fig. 3.4A, red cells treated with the vehicle (10 μl DMSO in 2 ml red cell suspension) for 2 hr at 37°C retained their normal biconcave appearance, though occasional small protuberances were observed in some of the cells. These protuberances were not seen in cells incubated in

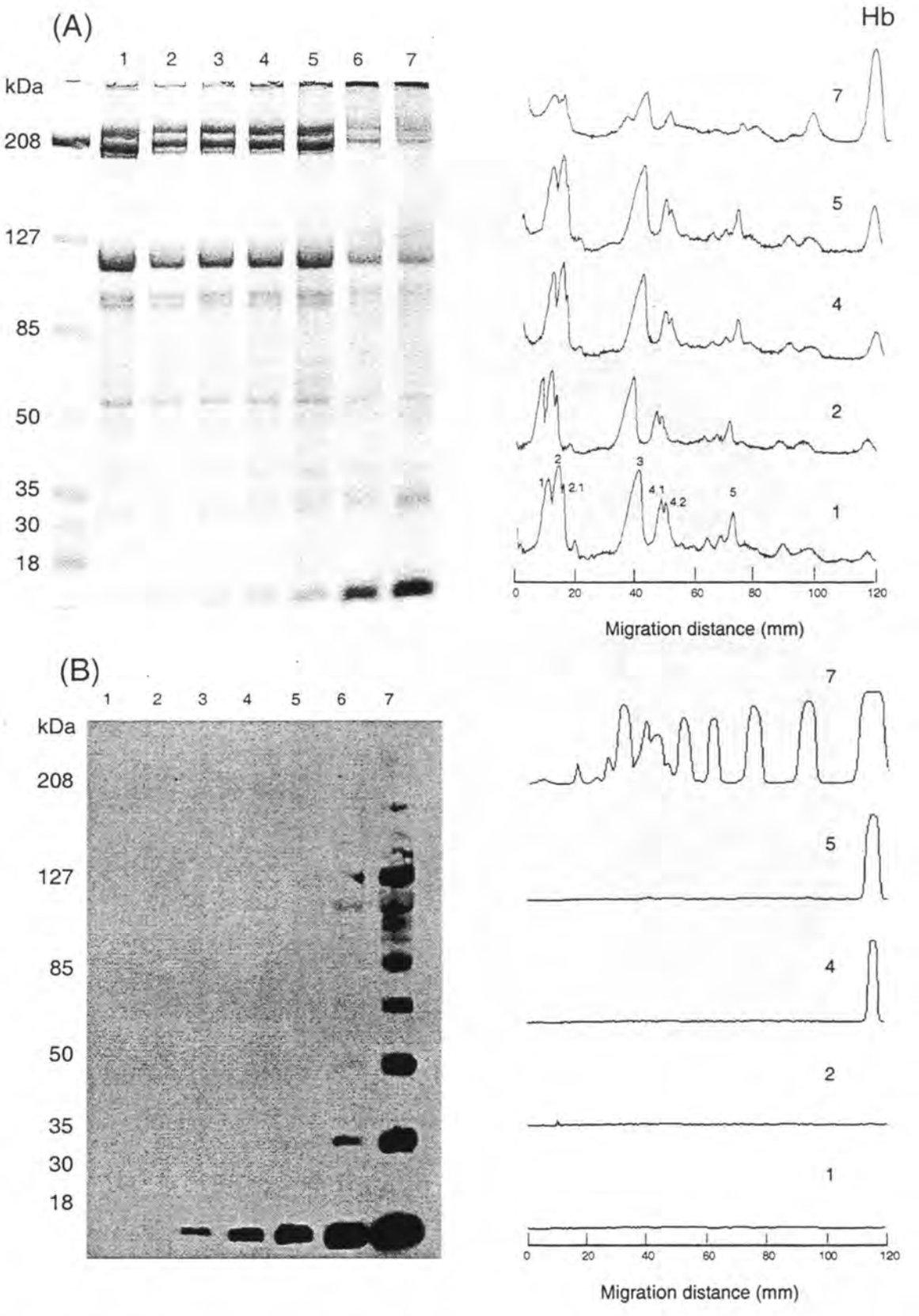

Fig. 3.3. Effect of MAQ-NOH on rat erythrocyte membrane skeletal proteins. Rat erythrocytes were incubated for 2 hr at 37°C in the presence of the vehicle (lane 1), 150 μM (lane 2), 350 μM (lane 3), 750 μM (lane 4), and 1.0 mM (lane 5) MAQ-NOH and 130 μM (lane 6) and 250 μM (lane 7) DDS-NOH. The cells were washed and membrane ghost were prepared and washed extensively to remove the unbound hemoglobin. The ghosts (32 μg protein) were the solubilized in SDS and subjected to PAGE. A) Gel Code blue stained gel and densitometric scans; B) immunoblot stained with rabbit anti-rat hemoglobin. The protein bands were identified according to Fairbanks *et al.* (1971).

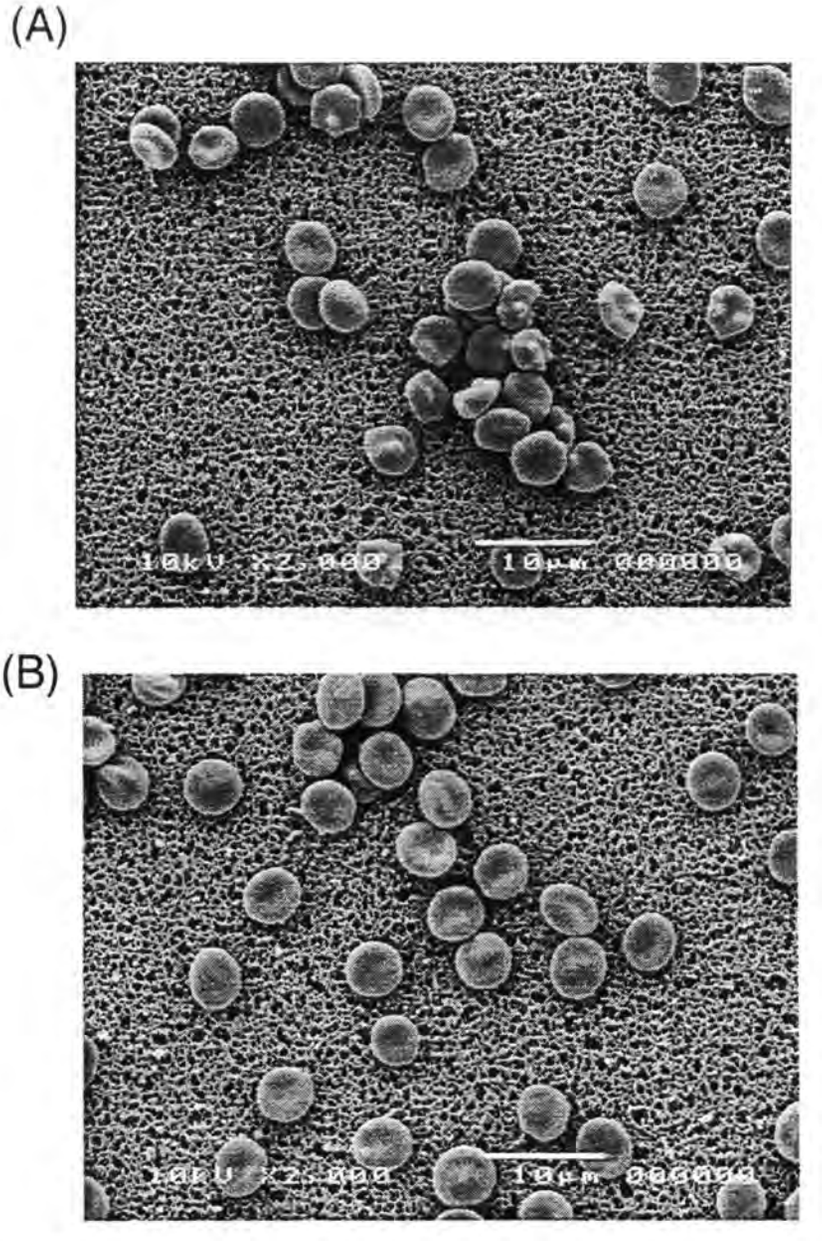

Fig. 3.4. Effect of MAQ-NOH on rat erythrocyte morphology. Scanning electron micrograph of (A) rat erythrocytes incubated for 2 hrs at 37°C in PBSG containing the vehicle; and (B) rat erythrocytes exposed to MAQ-NOH (1mM). Magnification X2000.

normal saline alone and are attributed to the DMSO. Rat erythrocytes incubated with MAQ-NOH, even at a very high concentration (1 mM; fig. 3.4B), also exhibited normal discocytic morphology.

Effect of MAQ-NOH on GSH-Depleted Erythrocytes

The enhanced sensitivity of red cells from G6PD-deficient individuals to the oxidative actions of certain drugs is considered to be due to their diminished capacity to maintain sufficient levels of NADPH, and hence GSH, when challenged by the oxidant stress. In an effort to reproduce this enhanced sensitivity in normal rat red cells, GSH was depleted by >90% using DEM prior to MAQ-NOH exposure. As shown in fig. 3.5, the hemolytic activity of MAQ-NOH (250 μM) was increased markedly in GSH-deficient red cells (T_{50} = 0.85 ± 0.06 days) as compared to MAQ-NOH-treated red cells with normal GSH levels (T_{50} = 7.4 ± 0.6 days). Survival of red cells treated with DEM alone (T_{50} = 8.6 ± 0.8 days) was not significantly different from the controls (T_{50} = 9.5 ± 0.9 days). As shown in Table 3.1, GSH depletion did not increase the extent of lipid peroxidation (TBARS formation) in MAQ-NOH treated red cells, indicating that the enhanced toxicity was not due to exacerbation of lipid peroxidation.

SDS-PAGE analysis of the skeletal proteins (fig. 3.6A) revealed an increase in hemoglobin monomer present in GSH-deficient red cells treated with MAQ-NOH (lanes 2-4) as compared to the GSH-normal MAQ-NOH control (lane 1). Immunoblot analysis using an anti-hemoglobin antibody (fig. 3.6B) showed that in the GSH-depleted cells, MAQ-NOH also caused a concentration-dependent increase in the formation of hemoglobin adducts with the membrane skeletal proteins (spectrin, ankyrin, bands 3 and

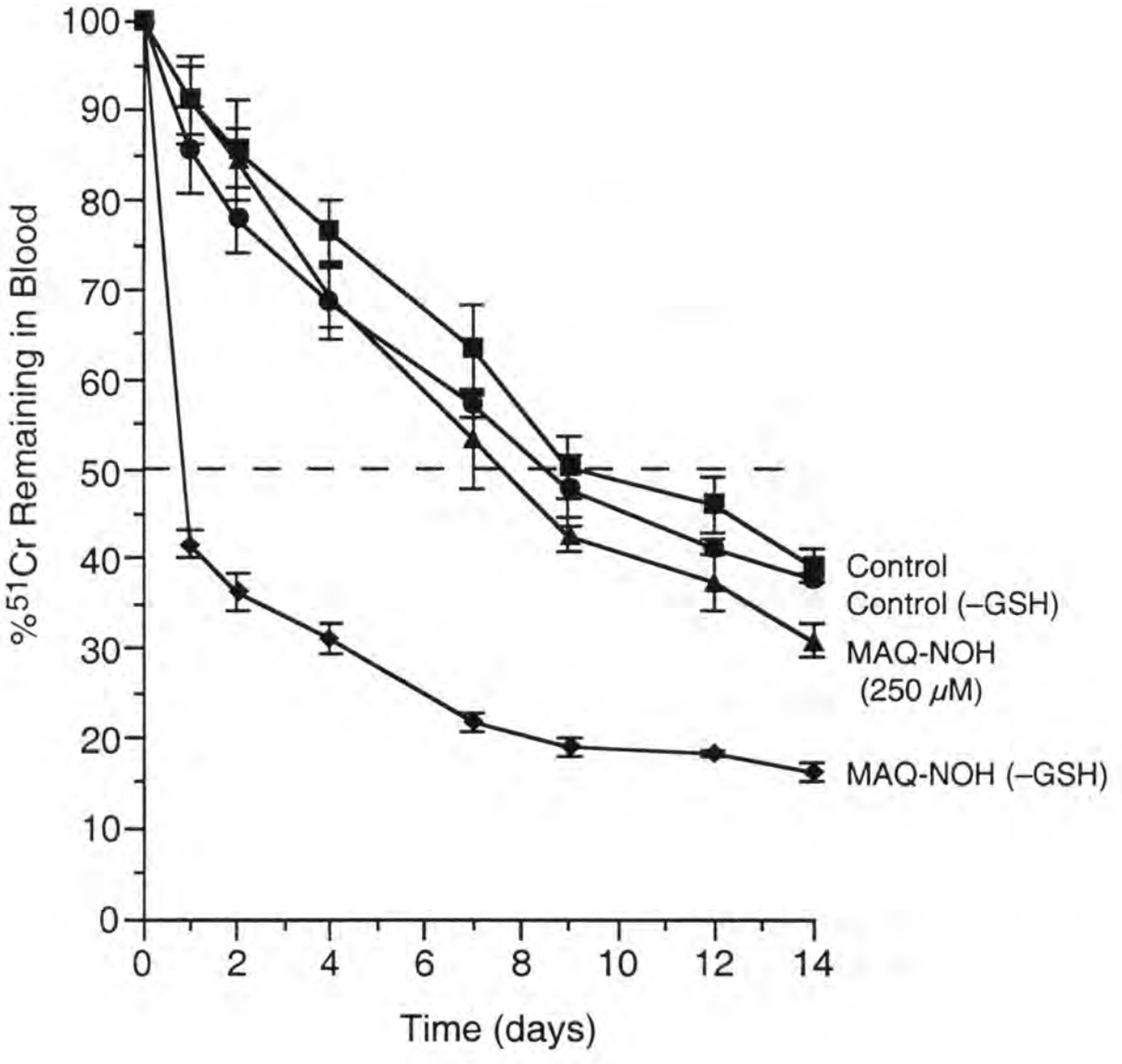

Fig. 3.5. The survival of GSH depleted ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure of 250 μM MAQ-NOH. Radiolabeled red cells were treated with DEM to depleted intracellular GSH (>90%). The cells were then incubated with vehicle or 250 μM MAQ-NOH for 2 hr at 37°C. The erythrocytes were then washed and administered i.v. into a group of isologous rats. T_o blood samples were taken 30 min after administration of the labeled cells. Data points are means $\pm$ SD (n=4).

Table 3.1. Effect of GSH Depletion on MAQ-NOH-induced TBARS Formation

Treatment	TBARS (μM)
Vehicle	0.155 ± 0.182
MAQ-NOH	0.352 ± 0.106*
MAQ-NOH -GSH	0.248 ± 0.148*
Phenylhydrazine	1.133 ± 0.166*

Rat erythrocytes in PBSG were depleted of GSH (>90%) using DEM and incubated at 37°C for 60 min in vehicle (DMSO), MAQ-NOH (250 μM), or Phenylhydrazine (5 mM). After the incubation, the cells were lysed, centrifuged, and analyzed for TBARS as described in "Materials and Methods". The values are means ± SD (n=3). *Significantly different from control ($p>0.05$).

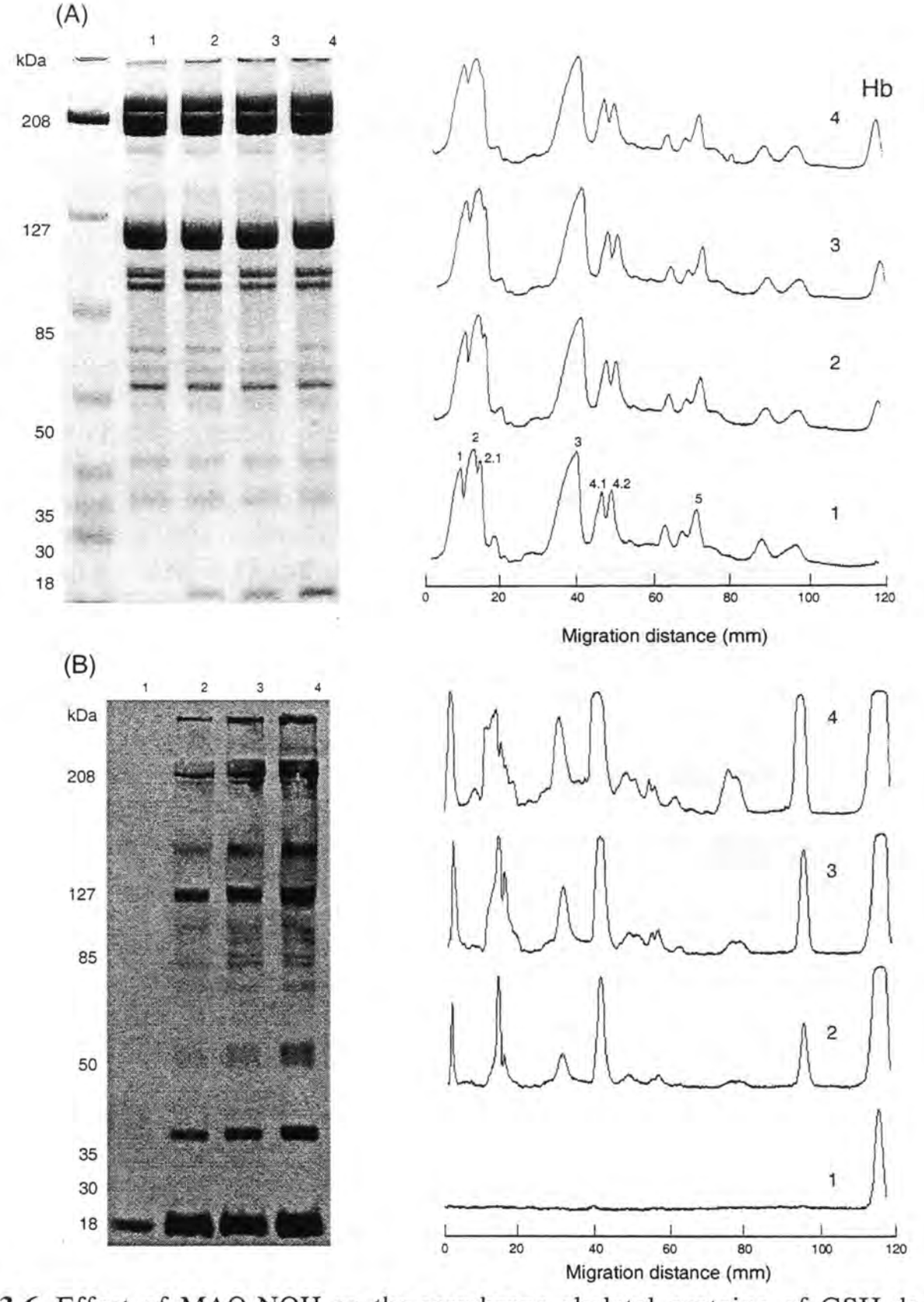

Fig. 3.6. Effect of MAQ-NOH on the membrane skeletal proteins of GSH-depleted erythrocytes. GSH depleted (90%) rat erythrocytes were incubated in the presence of DMSO (lane 1), 150μM MAQ-NOH (lane 2), 250μM MAQ-NOH (lane 3), or 350μM MAQ-NOH (lane 4) for 2 hr at 37°C. Following exposure, the cells were washed and membrane ghosts were prepared and washed extensively to remove any unbound hemoglobin. The ghosts (32μg of protein) were solubilized in SDS and subjected to PAGE. A) Gel Code Blue stained gel B) immunoblot stained with rabbit anti-rat hemoglobin. The proteins were identified according to Fairbanks *et al.* (1971).

4.2). The increase in membrane-bound hemoglobin was observed in the form of high molecular weight aggregates (>100 kDa) and in hemoglobin monomers and dimers (lanes 2-4). These data indicate that in GSH-deficient red cells, skeletal protein becomes a preferential target of the oxidant action induced by MAQ-NOH.

Discussion

The present results demonstrate that under *in vitro* incubation conditions known to commit rat erythrocytes to premature removal from the circulation after their re-administration to isologous animals, MAQ-NOH induced alterations in red cell sulfhydryl status (fig. 3.1) and caused damage to the plasma membrane in the form of lipid peroxidation (fig. 3.2). Overall, the data presented here support the concept that oxidative stress underlies MAQ-NOH hemolytic activity.

On the one hand, the ability of MAQ-NOH to deplete GSH was not unexpected in view of our previous studies with other arylhydroxylamines, and as with these other agents, the loss of GSH could be accounted for by oxidation to GSSG and GS-protein. However, examination of the time-course (fig. 3.1A) and concentration dependence (fig. 3.1B) for MAQ-NOH-induced alterations in sulfhydryl status indicated that there are striking differences between this compound and previously examined N-hydroxy compounds. For example, with DDS-NOH, hemolytic activity is associated with a rapid, extensive and long-lasting depletion of red cell GSH (Grossman et al., 1992). In the case of MAQ-NOH, the loss of GSH was transient and relatively modest across the full range of hemolytic concentrations. These data indicate that the MAQ-NOH concentration-response curve for GSH oxidation (EC_{50} ca. 1000 μM) is well to the right of the curve for the hemolytic response, which has a TC_{50} of about 350 μM and exhibits a maximal

response at about 750 μM (Bolchoz et al., 2001). This relationship suggests that while MAQ-NOH does cause depletion of red cell GSH, the depletion of cellular GSH *per se* is not a prerequisite for the oxidative stress-mediated hemolytic damage that MAQ-NOH inflicts on the red cell.

On the other hand, MAQ-NOH-induced lipid peroxidation was unexpected in view of the lack of lipid peroxidation observed previously in red cells treated with hemolytic concentrations of DDS-NOH (McMillan et al., 1998). Lipid peroxidation was detected in MAQ-NOH-treated red cells using two independent measurements, TBARS and F_2-isoprostanes (fig. 3.2), and occurred across the range of hemolytic concentrations of MAQ-NOH. In contrast, cytoskeletal protein thiol oxidation, as indicated by formation of hemoglobin-skeletal protein adducts, was not significant after exposure of red cells to hemolytic concentrations of MAQ-NOH (fig. 3.3). These data suggested that skeletal protein is not the preferred intracellular target of MAQ-NOH, as it is for DDS-NOH.

It should be noted that although skeletal protein was apparently not affected by MAQ-NOH, there was a concentration-dependent increase in the amount of hemoglobin monomer present in membrane ghosts prepared from MAQ-NOH-treated red cells (fig. 3.3). This monomer could not be removed from the ghosts by repetitive washing and is presumed to be bound either non-covalently (hydrophobic interaction) or covalently to the lipid matrix. The molecular form of this membrane-bound hemoglobin and its significance remain to be determined. Nevertheless, binding of the monomer may be a key event in the hemolytic process because it is observed with other arylhydroxylamines, such as DDS-NOH (Grossman et al., 1992), and has been seen with other hemolytic agents, such as divicine (McMillan et al., 2001), as well as in rat red cells aged to

senescence by the serial hypertransfusion technique (McMillan and Jollow, unpublished observations).

Alterations in erythrocyte morphology were notably absent in MAQ-NOH treated red cells (fig. 3.4). In view of the well known role of the skeletal protein assembly in the maintenance of red cell shape (Marchesi, 1985), the lack of gross morphological changes in these red cells is consistent with the absence of changes in the electrophoretic mobility of the skeletal proteins on SDS-PAGE gels (figs. 3.3 and 3.6). The lack of morphological change after hemolytic concentrations of MAQ-NOH is in sharp contrast to the dramatic changes in erythrocyte morphology observed with other hemolytic agents. For example, hemolytic concentrations of DDS-NOH and divicine induce distinctive echinocytic morphology (Grossman et al., 1992; McMillan et al., 2001), and phenylhydrazine is well known to cause transformation of red cells to spheroechinocytes (Rifkind and Danon, 1965).

Although the data presented here support an oxidative stress-type mechanism for MAQ-NOH hemotoxicity, its low potency as a GSH-depleting agent made us wonder whether cells lacking GSH (or the ability to replenish it) would be more sensitive to MAQ-NOH induced oxidant damage. Furthermore, we reasoned that since lipid peroxidation appeared causal in the hemolytic process whereas protein oxidation was not, then any enhancement in hemotoxicity provoked by depletion of GSH should be accompanied by corresponding increases in lipid peroxidation without affecting the level of protein oxidation. When red cell GSH was depleted (>90%) by titration with DEM prior to exposure to MAQ-NOH, the hemolytic activity of MAQ-NOH was markedly

enhanced (fig. 3.5). Surprisingly, however, exacerbation of hemolytic activity was not accompanied by an increase in lipid peroxidation (Table 3.1).

Although the SDS-PAGE pattern of the skeletal proteins was not visibly changed in MAQ-NOH-treated, GSH-deficient red cells (fig. 3.6A), examination of the skeletal proteins by immunoblotting revealed the presence of hemoglobin-skeletal protein adducts. Moreover, the presence of these adducts in GSH-deficient red cells was dependent on MAQ-NOH concentration (fig. 3.6B). The fact that the amount of hemoglobin adducted to the skeletal protein was insufficient to perturb the SDS-PAGE pattern suggests that it is quantitatively much less than that seen with equally hemotoxic concentrations of a "pure" protein thiol oxidizer, such as DDS-NOH. This raises the possibility that protein oxidation and lipid peroxidation act additively, and perhaps even synergistically, in initiating the intracellular events that lead to premature splenic sequestration. However, it should be noted that the quantitative relationship between the initial "hit" and commitment of the cells to removal is not yet known. The possibility that MAQ-NOH may operate by multiple mechanisms in the GSH-depleted red cell, and that these mechanisms may be synergistic, is intriguing and warrants further investigation.

We have shown previously that N-hydroxylamines are responsible for the hemolytic activity observed after administration of several arylamine drugs and environmental chemicals, including aniline (Harrison and Jollow, 1986), dapsone (Grossman and Jollow, 1988), phenacetin (Jensen and Jollow, 1991) and propanil (McMillan et al., 1991). Although the mechanism by which these N-hydroxylamines cause hemolytic injury is not completely understood, evidence suggests that these compounds produce damage within red cells as a consequence of their coupled oxidation with

oxyhemoglobin, yielding methemoglobin and the arylnitroso derivative. In the course of this reaction, reactive oxygen species, thiyl radicals and possibly other (i.e., compound-centered) free radicals are generated (Kiese, 1974; Maples et al., 1990; Bradshaw et al., 1997). It is believed that these free radicals oxidize critical sites within the red cell that ultimately transmit a signal to the external cell surface, marking the cell for removal from circulation by macrophages. The precise internal lesion responsible for premature sequestration of these cells, whether on lipid or protein, is still not known.

One theory is that free radical induced membrane lipid peroxidation may play a role in transmitting a signal for removal. Jain et al. (Jain, 1984) have shown that the lipid peroxidation byproduct malondialdehyde can flip phosphatidyl serine from the inner to the outer leaflet of the plasma membrane. Disruption of the asymmetrical distribution of phosphatidyl serine in the plasma membrane has been shown to stimulate erythrophagocytosis (Tanaka and Schroit, 1983; McEvoy et al., 1986; Bonomini et al., 2001). A second postulate suggests that disulfide-linked hemoglobin adducts to critical membrane skeletal proteins, formed via hemoglobin thiyl radical attack of the protein free sulfhydryl groups, initiates red cell sequestration (Jollow et al., 1995; Jollow and McMillan, 1998). In support of this postulate, a number of investigators have shown that hemoglobin attachment to the cytosolic domain of band 3 causes alterations in the lateral distribution of this integral membrane protein leading to the binding of autologous antibodies on the external cell surface, which initiates erythrophagocytosis (Lutz et al., 1984; Waugh et al., 1986; Waugh et al., 1987; Turrini et al., 1991).

In conclusion, assessment of the contribution that MAQ-NOH makes towards the hemotoxicity of primaquine is difficult. One problem is the multiplicity of unstable,

redox-active metabolites that are formed during the metabolic clearance of primaquine. These include several phenolic derivatives that have been implicated by others in the hemotoxicity of primaquine (Link et al., 1985; Augusto et al., 1988; Fletcher et al., 1988; Agarwal et al., 1991). Thus, it is conceivable that primaquine hemotoxicity in humans is mediated by more than one type of toxic metabolite.

Secondly, there may be interactions among these metabolites which facilitate the development of oxidative damage within the erythrocyte. For example, preliminary observations in our laboratory indicate that a phenolic metabolite, 5-hydroxyprimaquine, is also a direct-acting hemolytic agent. Furthermore, in contrast to MAQ-NOH, 5-hydroxyprimaquine is also a potent GSH-depleting agent (McMillan et al., unpublished studies). This observation suggests that by depleting GSH, 5-hydroxyprimaquine may sensitize the red cell to the hemolytic activity of MAQ-NOH. If so, this effect would be amplified in G6PD-deficient red cells due to their already reduced levels of GSH. As noted by Degowin et al. (Degowin et al., 1966), G6PD-deficient individuals are about 20-30 fold more sensitive to the hemolytic activity of primaquine than are G6PD-normal individuals. Clearly, the possibility of metabolite synergism in primaquine-induced hemolytic anemia warrants further investigation.

CHAPTER 4

Primaquine-Induced Hemolytic Anemia: Formation of Free Radicals in Rat Erythrocytes Exposed to 6-Methoxy-8-Hydroxylaminoquinoline

Introduction

Hemolytic anemia and methemoglobinemia are well recognized dose-limiting side effects in the therapeutic use of arylamine drugs, such as primaquine and dapsone (Beutler, 1969). Because these compounds are not hemotoxic when incubated with red cells *in vitro*, it has long been appreciated that metabolites are responsible for onset of the hemolytic response. In the cases of aniline (Harrison and Jollow, 1986), dapsone (Grossman and Jollow, 1988), and phenacetin (Jensen and Jollow, 1991), we have shown that the hemolytic metabolites are their N-hydroxy derivatives. Primaquine metabolism, on the other hand, is relatively more complex and the metabolites which mediate the hemotoxic responses have not been identified. A variety of known and putative phenolic metabolites of primaquine are redox-active and therefore have the potential to mediate primaquine hemotoxicity (Strother et al., 1984; Baird et al., 1986; Fletcher et al., 1988; Agarwal et al., 1991); however, direct evidence for their hemolytic activity is lacking. We have recently explored an alternative hypothesis; that primaquine hemotoxicity is mediated by an N-hydroxylated metabolite, 6 methoxy-8-aminoquinoline (MAQ-NOH).

Although the mechanism underlying the damage and removal red cells by hemolytic N-hydroxylamines remains unclear, oxidative stress has long been considered to play a prominent role in the process (for review, see Beutler, 1971). This concept is based on the well-known association of hemotoxicity with oxidation of erythrocytic GSH (to GSSG and glutathione-protein mixed-disulfides), methemoglobin formation, and with

enhanced sensitivity to the hemolytic effect of these agents in individuals deficient in glucose-6-phosphate dehydrogenase (G6PD) activity.

Considerable evidence generated by Kiese and colleagues (Kiese, 1974) suggests that the oxidative stress provoked by N-hydroxylamines is due to a cyclic oxidation-reduction reaction involving the hydroxylamine, oxyhemoglobin, and molecular oxygen, to yield the nitrosoarene, methemoglobin, and partially reduced forms of oxygen, respectively. This interaction has been shown to produce greater than stoichiometric amounts of reactive oxygen species (i.e., hydrogen peroxide, superoxide anion radical, hydroxyl radical) and sulfur-centered free radicals (i.e., glutathione and hemoglobin thiyl radicals), and thus has long been considered to generate reactive species capable of causing cellular injury (Rostorfer and Cormier, 1957; Maples et al., 1990; Bradshaw et al., 1995; Bradshaw et al., 1997).

We have reported recently that an N-hydroxy metabolite of primaquine, MAQ-NOH, is a direct-acting hemolytic agent in rats, and hence may be a contributor to primaquine-induced hemolytic anemia (Bolchoz et al., 2001). Although the hemolytic response induced by this metabolite was generally similar to that of other structurally related hydroxylamines, we observed some marked differences in the oxidative activity of this metabolite in rat red cells (Bolchoz et al., 2002). Of interest, when hemolytic concentrations of MAQ-NOH were added to rat red cells, the depletion of cellular GSH and the formation of methemoglobin was significantly less than that seen with equally hemolytic concentrations of other arylhydroxylamines, implying a quantitative difference in the extent of oxidant stress and cellular injury. Furthermore, this metabolite induced concentration dependent lipid peroxidation without evidence of protein oxidation.

However, when red cell GSH was depleted by titration with diethyl maleate prior to MAQ-NOH exposure, there was a marked enhancement of hemolytic activity without a corresponding increase in lipid peroxidation. The enhanced hemotoxicity was accompanied instead by the appearance of protein oxidation in the form of disulfide-linked hemoglobin-skeletal protein adducts. We have hypothesized that free radicals, derived either from molecular oxygen or MAQ-NOH itself (i.e., a compound-centered free radical), are responsible for the damage to membrane lipids and skeletal proteins that marks the injured red cells for premature removal from the circulation by macrophages in the spleen.

Thus, the present studies were conducted to determine if free radicals could be detected and identified in rat red cell suspensions exposed to hemolytic concentrations of MAQ-NOH, and thus have a role in MAQ-NOH-induced hemolytic injury. MAQ-NOH was added to rat red cell suspensions, and the incubates were analyzed by UV-visible spectroscopy and EPR using the spin trap 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO). We report that MAQ-NOH was able to generate hydroxyl radicals and ferryl heme species under hemolytic conditions. Furthermore, formation of the hydroxyl radical was constant for twenty minutes and dependent on the presence of erythrocytic GSH. These data support the concept that oxygen free radicals are involved in the process underlying MAQ-NOH-induced hemolytic damage

Materials and Methods

Chemicals

Diethylenetriaminepentaacetic acid (DTPA) and diethyl maleate were obtained from Sigma Chemical Co. (St. Louis, MO). EMPO was purchased from Oxis Research (Portland OR). MAQ-NOH was synthesized as described previously (Allahyari et al., 1984). All other reagents were of the best commercially available grade.

Animals

Male Sprague-Dawley rats were purchased from Harlan Labs (Indianapolis, IN) and were maintained on food and water *ad libitum*. Animals were acclimated to a 12-hour light-dark cycle for one week prior to their use. Blood was collected from the descending aorta of anesthetized rats into heparinized tubes and washed three times in isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM D-glucose (PBSG). Following removal of the plasma and buffy coat, the cells were resuspended (40% hematocrit) and used the same day they were collected.

EPR studies

Reaction mixtures (2 ml) contained 10 mM EMPO, 0.1 mM DTPA in a red cell suspension (40%) in PBSG at room temperature under aerobic conditions. Experiments that utilized hemolyzed red cells were conducted by suspending wased red cells in ice-cold de-ionized water. Reactions were initiated by addition of MAQ-NOH dissolved in DMSO (10 μl). EPR spectra were recorded on a Bruker ELEXYS E-500-10/12 spectrometer system (Bruker Instruments, Inc., Billerica, MA) operating at 20 mW power with a microwave frequency of 9.77 GHz, a receiver gain of 69, and a time constant of 0.164 sec.

Stability of MAQ-NOH in buffer and in red cell suspensions was determined by HPLC with electrochemical detection as described previously (Bolchoz et al., 2001). For experiments to determine the dependence of radical adduct generation on red cell GSH, red cell suspensions were titrated with diethyl maleate to deplete GSH (by >95%) as described previously (Bolchoz et al., 2002).

Spectrophotometric detection of hemoglobin oxidation

Formation of methemoglobin was determined as described previously (Harrison and Jollow, 1987; Bradshaw et al., 1997). To determined if MAQ-NOH induced ferrylhemoglobin formation in rat red cells (Harrison and Jollow, 1987; Bradshaw et al., 1997), aliquots (75 μl) of MAQ-NOH-treated red cell suspensions were lysed in 5 ml of hemolysis buffer (5 mM sodium phosphate dibasic, 0.5% triton X-100, pH 7.5) and analyzed in a Shimadzu UV-160A double-beam UV-visible spectrophotometer. The amount of ferrylhemoglobin was measured in red cell incubates treated with sodium sulfide (2 mM) at 37°C for 5 min prior to MAQ-NOH exposure. Sodium sulfide irreversibly converts the unstable ferryl heme species to sulfhemoglobin which is measured spectrophotometrically at 620 nm (Giulivi and Davies, 1990).

Results

Formation of EMPO radical adducts in rat red cells

To establish whether free radicals could be detected in rat red cells exposed to MAQ-NOH under previously determined hemolytic conditions (Bolchoz et al., 2001), rat red cell suspensions (40% hematocrit) containing 10 mM EMPO were exposed to 350 μM

MAQ-NOH. As shown in figure 4.1, a four line (1:2:2:1) EMPO radical adduct signal was generated. The signal was dependent on the presence of MAQ-NOH (fig. 4.1B) and EMPO (fig. 4.1C), and was generated equally well in intact and lysed red cells (fig. 4.1 A and E). The nitrogen hyperfine splitting constants (Table 4.1) were identical to those of an EMPO hydroxyl radical adduct (Olive et al., 2000). As shown in figure 4.2, formation of the EMPO-OH was concentration-dependent, and occurred over a range of hemolytic concentrations of MAQ-NOH. No evidence for an EMPO-thiyl radical adduct or a compound-centered free radical was observed in these incubates.

Time dependence of hydroxyl radical formation

Previous EPR studies using the spin trap, 5,5-dimethyl-pyrroline N-oxide (DMPO), have demonstrated that glutathione thiyl radical adducts are generated in rat red cell suspensions exposed to hemolytic concentrations of the arylhydroxylamine, phenylhydroxylamine (Maples et al., 1990; Bradshaw et al., 1995). These experiments also revealed that at higher drug concentrations and longer incubation times, the glutathione thiyl radical adduct signal was replaced by a hemoglobin thiyl radical adduct signal. In the present study, when rat red cells containing EMPO were exposed to MAQ-NOH (350 μM) and the spectrum recorded for 30 min, no change in the shape of the hydroxyl radical signal was detected (fig. 4.3). However, the signal intensity began to decline after 20 min., due to decay.

To investigate whether the production of the hydroxyl radical was constant with time, red cell suspensions were incubated with MAQ-NOH (350 μM) and EMPO was added to

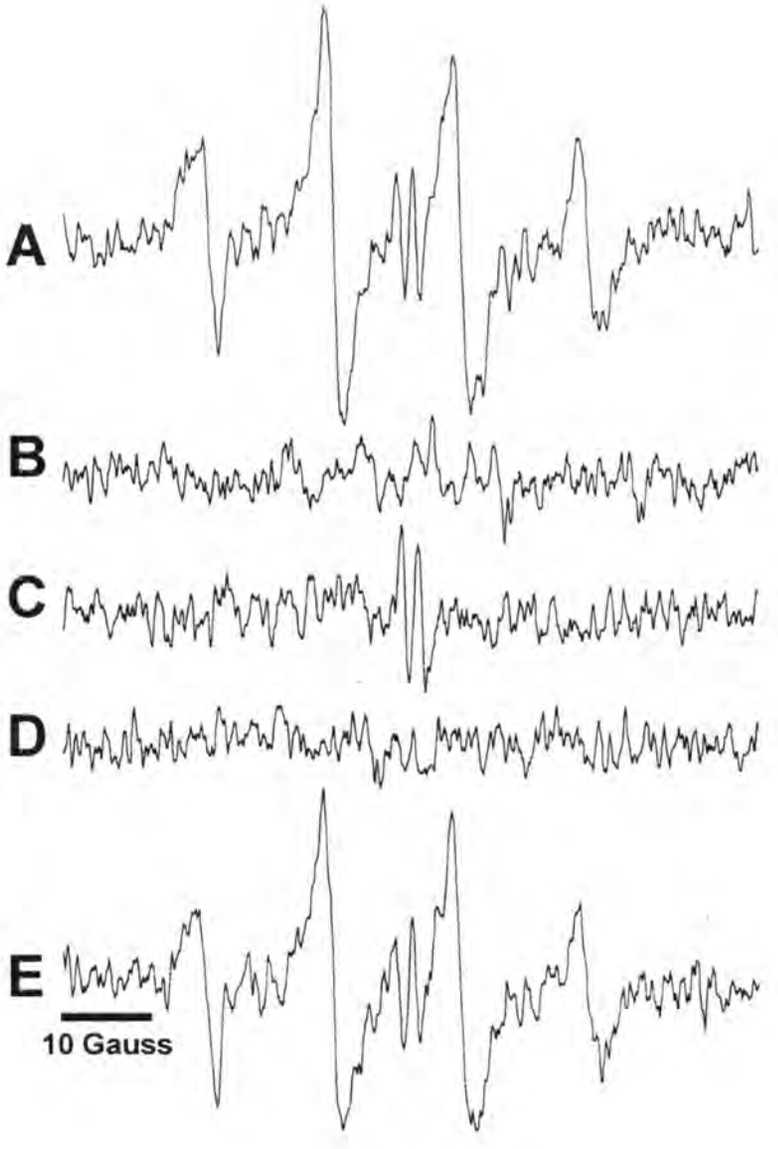

Fig. 4.1. Detection of EMPO-radical adduct signal in rat erythrocytes exposed to MAQ-NOH. (A) EPR spectrum recorded 3 min after the addition of MAQ-NOH (350 μM) to a 40% red cell suspension in PBSG containing 10 mM EMPO and 0.1 mM DTPA. (B) As in A, except MAQ-NOH was omitted. (C) As in A, except EMPO was omitted. (D) As in A, except red cells were omitted. (E) As in A, except lysed red cells were used.

Table 4.1. Hyperfine splitting constants of EMPO Hydroxyl Adduct Diastereomers

Spin Adduct	A_N/G	$A_{H\beta}$/G	$A_{H\gamma}$/G
EMPO-OH[a]	14.0	15.1	0.9
	14.0	12.7	
EMPO-OH[b]	13.94	14.97	0.73
	14.06	12.88	

[a]From Olive, 2000
[b]Splitting constants determined from EPR spectrum shown in figure 4.1A.

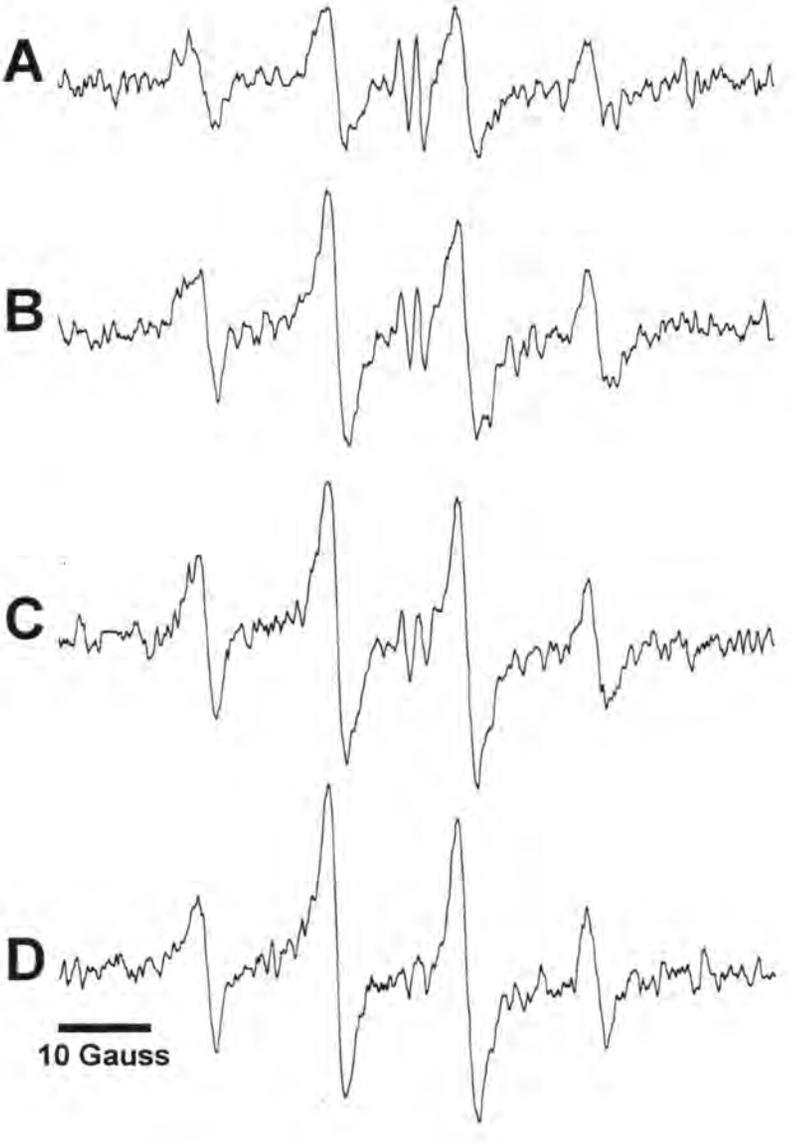

Fig. 4.2. Concentration-dependent increase in the MAQ-NOH-induced EMPO-OH signal in rat erythrocytes. EPR spectrum recorded 3 min after the addition of (A) 150 μM, (B) 350, (C) 750 μM, or (D) 1.5 mM MAQ-NOH to 40% red cell suspension in PBSG containing 10 mM EMPO and 0.1 mM DTPA.

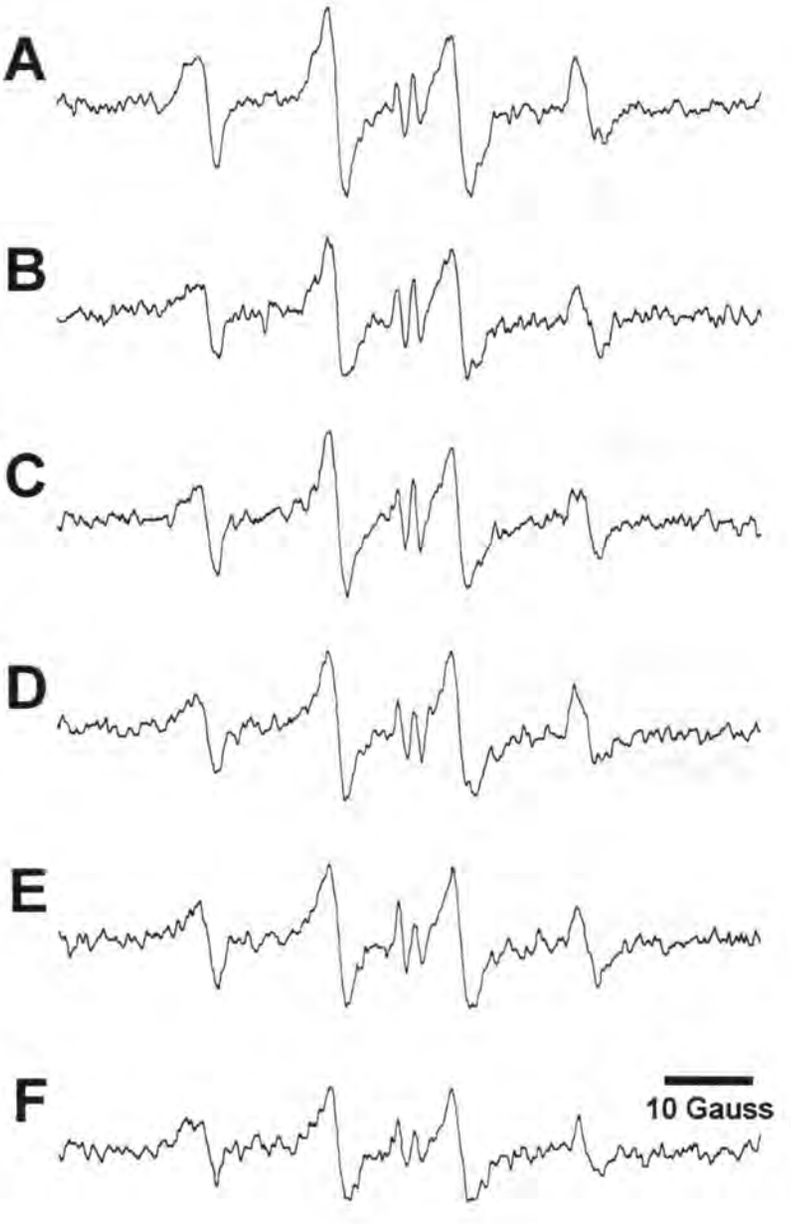

Fig. 4.3. Time dependence of MAQ-NOH-induced EMPO-radical adduct signal in rat erythrocytes. EPR spectrum recorded (A) 1 min, (B) 3 min, (C) 6 min, (D) 10 min, (E) 15 min, and (F) 30 min after the addition of MAQ-NOH (350 μM) to 40% red cell suspension in PBSG containing 10 mM EMPO and 0.1 mM DTPA.

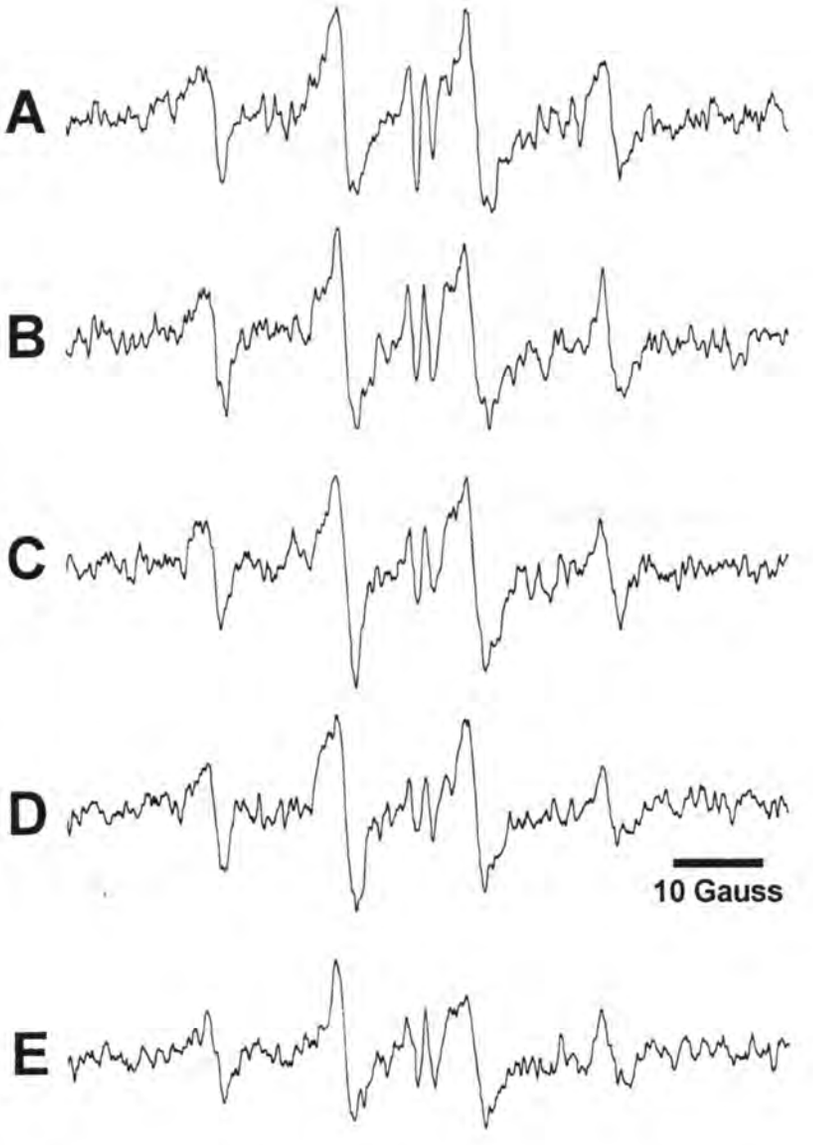

Fig. 4.4. Effect of time of EMPO addition on the MAQ-NOH-induced EPR signal. 10 mM EMPO was added to a 40% red cell suspension in PBSG containing 0.1 mM DTPA (A) 1 min, (B) 5 min, (C) 10 min, (D) 20 min, (E) 30 min after the addition of MAQ-NOH (350 μM). EPR spectrum recorded 3 min after the addition EMPO.

the mixture after 1, 5, 10, 20, and 30 min. As seen in figure 4.4, the hydroxyl radical adduct intensity was independent of the time the trap was added up to 20 min, after which the signal intensity began to decline.

To determine the relative stability of MAQ-NOH in buffer (PBSG) vs. red cells, the concentration of MAQ-NOH was measured in aliquots of the incubates by HPLC-EC. In the presence of red cells at 37°C, MAQ-NOH was highly unstable and with a half-life of about 2 min (data not shown). In contrast, when incubated in buffer alone, MAQ-NOH was relatively stable, disappearing with a half-life of about 4 min.

Effect of GSH depletion on hydroxyl radical formation

Recent work in this laboratory has shown that MAQ-NOH hemolytic activity is markedly enhanced in rat red cells depleted of reduced glutathione (>90%) (Bolchoz et al., 2002). To examine the effect of GSH depletion on hydroxyl radical formation, rat red cells were depleted of GSH (>95%) by titration with diethyl maleate prior to their exposure to MAQ-NOH. As shown in figure 4.5, hydroxyl radical adducts were not detected in suspensions that lacked GSH, even using concentrations of MAQ-NOH as high as 750 μM.

Ferrylhemoglobin formation in rat red cells

Ferrylhemoglobin is known to be generated in red cells by the interaction of oxyhemoglobin and H_2O_2, and is considered to be a potent cytotoxic oxidant capable of peroxidation of unsaturated fatty acids (Kanner and Harel, 1985b; Kanner and Harel, 1985a; Galaris et al., 1990). Since hemolytic concentrations of MAQ-NOH induce lipid

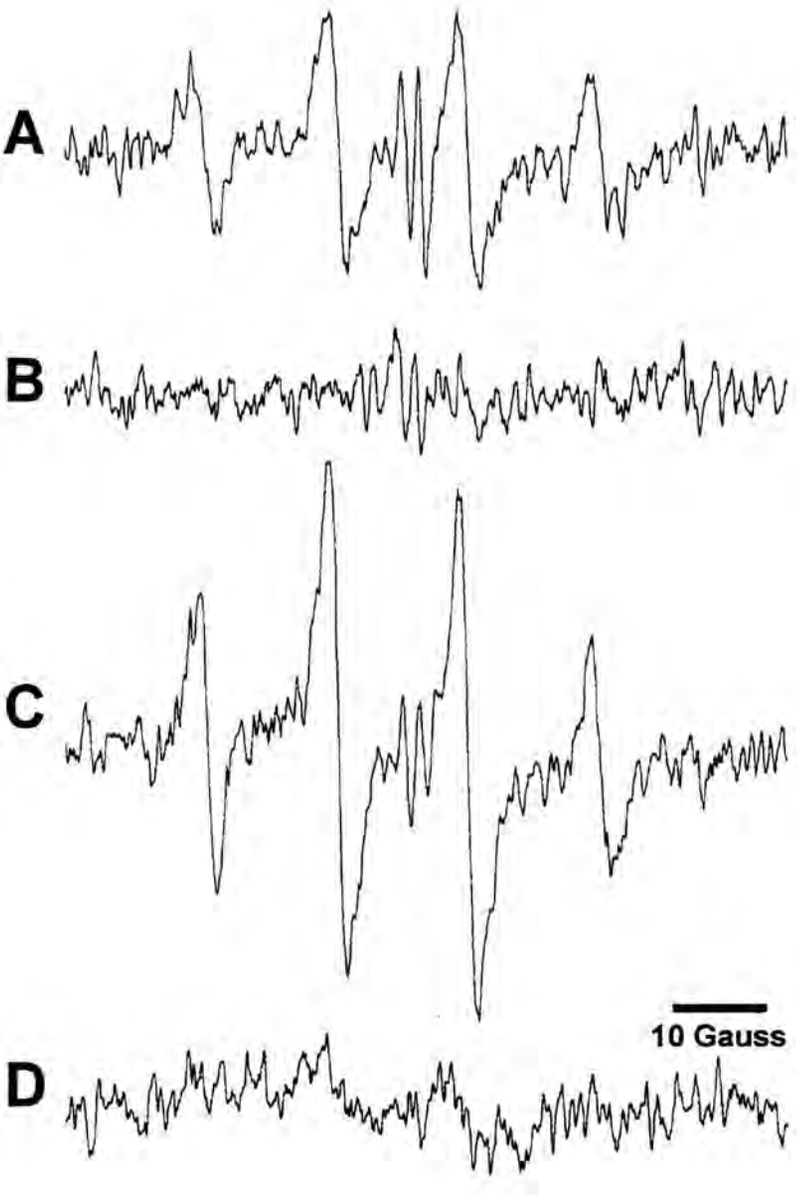

Fig. 4.5. Effect of removal of GSH from rat erythrocytes on the MAQ-NOH generated EPR signal. (A) EPR spectrum recorded 3 min after the addition of 150 μM MAQ-NOH to 40% red cell suspension in PBSG containing 10 mM EMPO and 0.1 mM DTPA. (B) As in A, except red cells were treated with DEM to deplete GSH (>95%). (C) As in A, except 750 μM MAQ-NOH was added. (D) As in C, except red cells were treated with DEM to deplete GSH (>95%).

peroxidation in rat red cells (Bolchoz et al., 2002), it was of interest to determine if ferrylhemoglobin was formed under the conditions used to detect free radical generation by EPR. Spectrophotometric scans of rat red cells exposed to hemolytic concentrations of MAQ-NOH (fig. 4.6) did not reveal the presence of ferrylhemoglobin as measured by increased absorbance at 545 and 580 nm. However, when the rat red cells were pretreated with Na_2S to trap ferryl heme species as sulfhemoglobin (Berzofsky et al., 1971), the presence of ferrylhemoglobin could be demonstrated. The fact that ferrylhemoglobin was detectable only by accumulation of sulfhemoglobin suggests a steady low level production of this oxidant species during the incubation period. Production of ferryl heme was dependent on MAQ-NOH concentration (fig. 4.7A) and on incubation time (fig. 4.7B).

Effect of GSH depletion on ferrylheme and methemoglobin formation

Since the enhancement of MAQ-NOH hemolytic activity caused by depletion of cellular GSH was accompanied by loss of the hydroxyl radical signal, it was of interest to determine if the enhanced toxicity was accompanied by ferrylhemoglobin formation. GSH-normal and GSH-depleted rat red cells were treated with a range of hemolytic concentrations of MAQ-NOH and assayed for ferrylhemoglobin. As shown in figure 4.8A, depletion of cellular GSH prior to addition of MAQ-NOH had no effect of the formation of ferryl heme in the red cells. Since ferrylhemoglobin is known to undergo a comproportionation reaction with oxyhemoglobin to yield molecules of methemoglobin (Giulivi and Davies, 1990), we also examined the effect of GSH depletion and of Na_2S pretreatment on MAQ-NOH-induced methemoglobin levels. As shown in figure 4.8B,

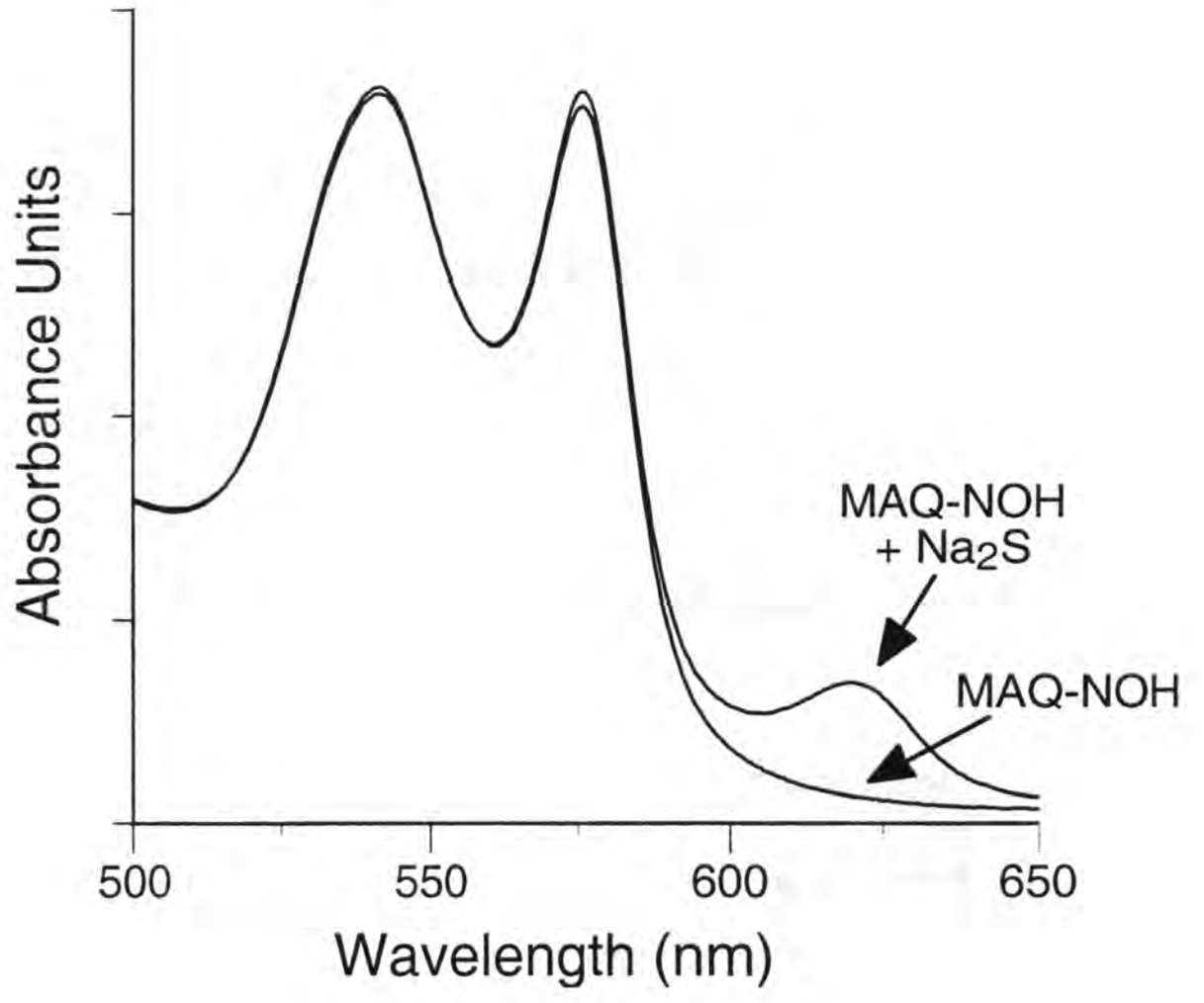

Fig. 4.6. Spectrophotometric detection of ferrylhemoglobin in rat erythrocytes exposed to MAQ-NOH. Rat erythrocytes (40% suspension) were pre-incubated with sodium sulfide (2 mM) to convert the ferryl heme species to sulfhemoglobin (λmax 620 nm) and then exposed to 750 μM MAQ-NOH for 30 min at 37°C. 10% Potassium cyanide (20 μl) was added to remove any absorbance interference due to methemoglobin.

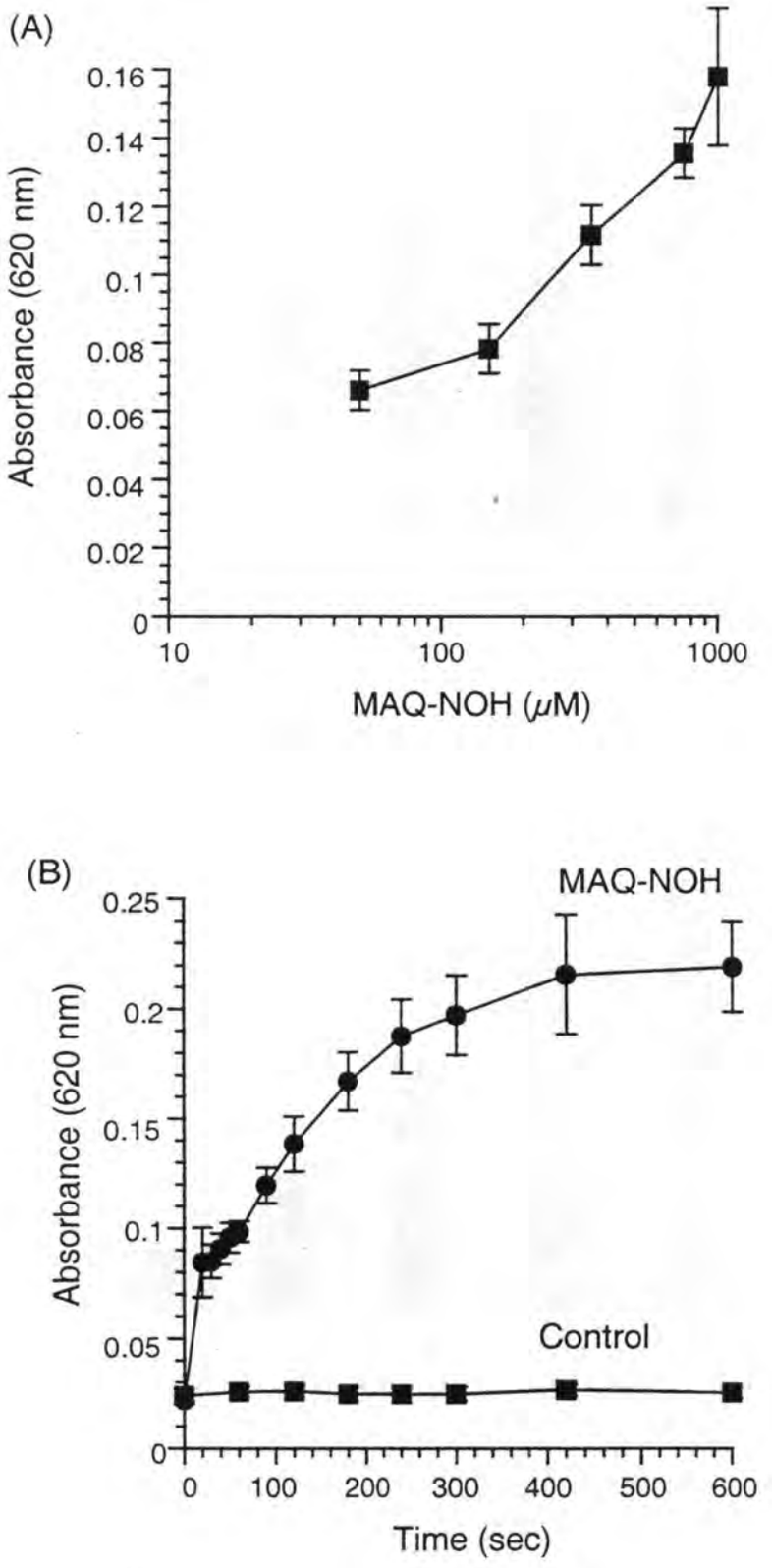

Fig. 4.7. Effect of concentration and time on MAQ-NOH-induced ferrylhemoglobin formation. (A) Absorbance at 620 nm 10 min after the addition of various hemolytic concentrations of MAQ-NOH to red cell incubates containing 2 mM sodium sulfide. 10% Potassium cyanide (20 μl) was added to remove any absorbance interference due to methemoglobin. (B) As in A, except red cells were incubated in 350 μM MAQ-NOH and aliquots taken at various time points.

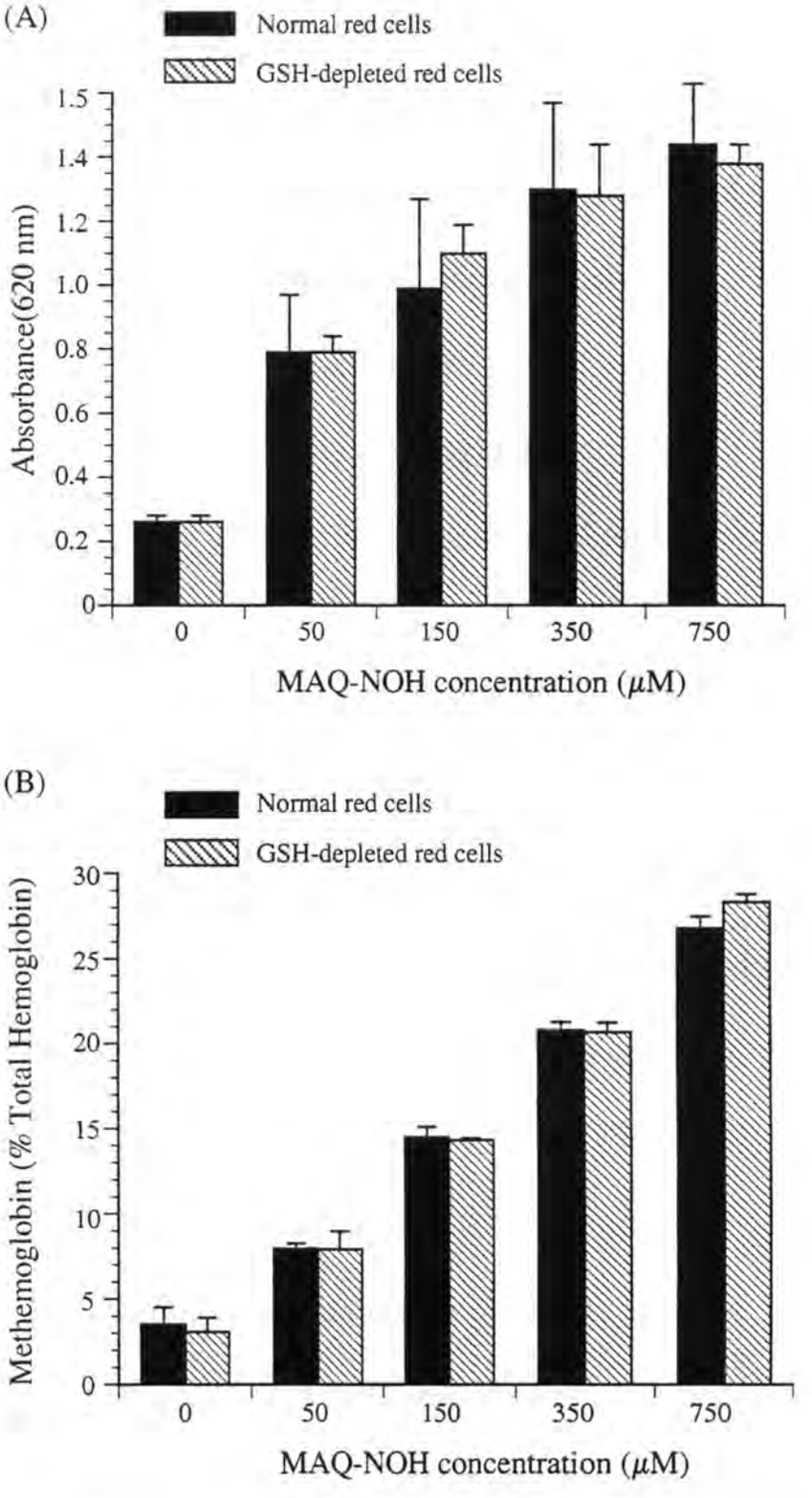

Fig. 4.8. Effect of GSH depletion on MAQ-NOH-induced (A) ferrylhemoglobin formation. Absorbance was recorded at 620 nm 10 min after the addition of various hemolytic concentrations of MAQ-NOH to red cell incubates containing 2 mM sodium sulfide. GSH-depleted red cells were treated with DEM prior to MAQ-NOH exposure. 10% Potassium cyanide (20 μl) was added to remove any absorbance interference due to methemoglobin. Data points are means ± SD (n=6). (B) As in A, except methemoglobin was determined as described previously (Harrison and Jollow, 1987). Data points are means ± SD (n=4).

depletion of GSH from the rat red cells also had no effect on the formation of methemoglobin.

Since inclusion of Na_2S in the incubation medium acts to trap ferryl heme as it is formed, comparison of methemoglobin levels in the presence and absence of Na_2S is a measure of the contribution of this pathway to methemoglobin formation induced by MAQ-NOH. As shown in figure 4.9, methemoglobin formation was unaffected by the presence of Na_2S, indicating that ferryl heme is not a major contributor to MAQ-NOH-induced methemoglobin formation.

Discussion

In previous studies on primaquine-induced hemolytic anemia, we demonstrated that its N-hydroxy metabolite, MAQ-NOH, is directly hemolytic in rats and that this hemotoxicity is associated with lipid peroxidation without significant protein oxidation (Bolchoz et al., 2001, Bolchoz, 2002 #306). However, when GSH is depleted prior to MAQ-NOH exposure, there is a pronounced exacerbation of MAQ-NOH hemolytic activity, and this exacerbation is accompanied by the appearance of protein oxidation without change in lipid peroxidation. The present studies were undertaken to determine if radical species are produced in rat red cells exposed to hemolytic concentrations of MAQ-NOH, and if so, whether the enhancement of hemolytic activity by GSH depletion is accompanied by alterations in free radical formation.

Experimentally, rat red cells were incubated with MAQ-NOH and analyzed by EPR using EMPO as the free radical spin trap. EMPO was utilized instead of the more widely used trapping agent, DMPO, because its superoxide radical adduct is more stable and

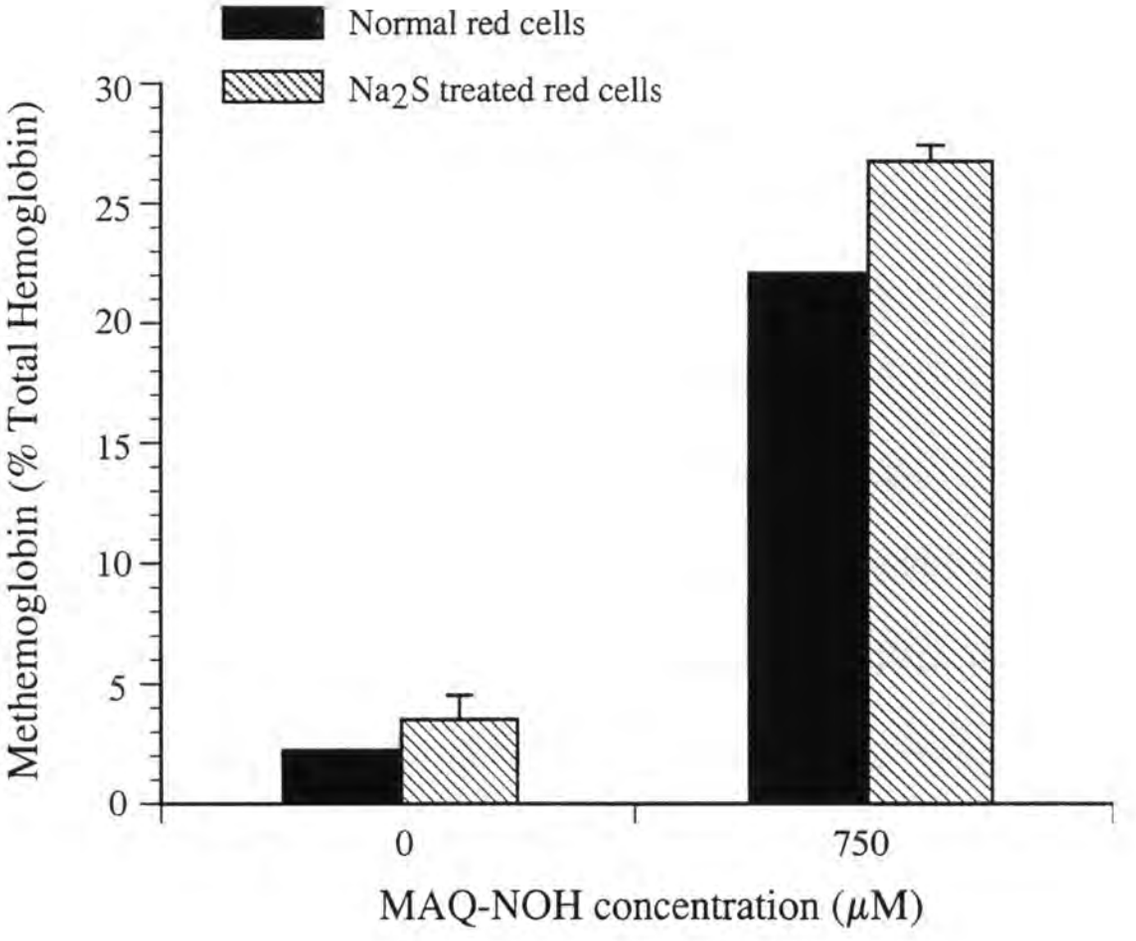

Fig. 4.9. Effect of Na_2S pretreatment on MAQ-NOH-induced methemoglobin formation. Red cell suspensions were incubated with 2 mM sodium sulfide or PBSG for 5 min prior to the addition of MAQ-NOH (750 μM). Methemoglobin was determined as described previously (Harrison and Jollow, 1987). Data points are means $\pm$ SD (n=3).

does not spontaneously decay to a hydroxyl radical adduct signal (Olive et al., 2000; Zhang et al., 2000), as is observed with the DMPO-superoxide adduct (Thornalley, 1986; Makino et al., 1990; Hanna et al., 1992). We report that MAQ-NOH generated an EMPO-hydroxyl radical adduct (fig 4.1) that was dependent on the presence of red cells (intact or lysed), EMPO, and MAQ-NOH. The EMPO-hydroxyl radical adduct signal was stable for 20 min (fig. 4.3) and its intensity was proportional to MAQ-NOH concentration (fig. 4.2). Addition of the EMPO reagent at intervals after exposure of the red cells to MAQ-NOH (fig. 4.4) generated a radical signal at a constant intensity for at least 20 min.

As noted above, depletion of red cell GSH prior to the addition of MAQ-NOH significantly enhances the hemolytic activity of MAQ-NOH. Paradoxically, hydroxyl radical was not only not enhanced, but was lost completely (fig. 4.5). Furthermore, the increased toxicity was not accompanied by an increase in ferryl heme formation (fig. 4.8A), and, hence by implication, not by increased levels of hydrogen peroxide.

Although a hydroxyl radical has previously been shown to be generated in red cells exposed to other hemolytic N-hydroxylamines, such dapsone hydroxylamine (Bradshaw et al., 1997), the mechanism by which the radical is generated is unclear. It is well known that oxyhemoglobin exists in the red cell as an equilibrium mixture, which includes a superoxide-ferriheme component. Dissociation of this complex is believed to be responsible for the constant, low-level oxidant stress that is present in unchallenged red cells. Kiese and colleagues (for review, see Kiese, 1974) proposed an interaction between arylhydroxylamines and oxyhemoglobin that may be regarded as an exacerbation of this normal process. Thus, they described the occurrence of a cyclic oxidation-reduction reaction between arylhydroxylamines and oxyhemoglobin, that yields the nitrosoarene,

methemoglobin, and a reactive oxygen species (such as superoxide or hydrogen peroxide), respectively. Subsequent reduction of methemoglobin by NADH-dependent methemoglobin reductase, and of the nitrosoarene by a NADPH-dependent diaphorase, would support a greater than one-to-one stoichiometry in regard to active oxygen production. Superoxide/hydrogen peroxide would in turn generate hydroxyl radicals by way of an iron-catalyzed Fenton reaction. Although there is controversy over whether hemoglobin is able to catalyze a Fenton reaction (Gutteridge, 1986; Puppo and Halliwell, 1988; Dong Mao et al., 1994), Comporti and colleagues (Ferrali et al., 1992; Ciccoli et al., 1994; Ciccoli et al., 1999) have presented evidence to suggest that oxidation of hemoglobin to methemoglobin facilitates the release of heme and/or free iron in a diffusible, redox-active form.

Although the MAQ-NOH-induced hydroxyl radical of the present studies could be generated by this mechanism, the loss of the hydroxyl radical when GSH was depleted from the red cells strongly suggests that GSH is involved in the process. An alternate hypothesis, proposed by Winterbourn and colleagues (Munday and Winterbourn, 1989; Winterbourn, 1993), is that superoxide formation is dependent on GSH. In this concept, GSH acts as a direct radical scavenger of a diverse range of radical species, accepting the unpaired electron from the radical to form a glutathione thiyl radical intermediate. The thiyl radical electron is then passed on to molecular oxygen to form superoxide anion radical, which under normal conditions is detoxified by superoxide dismutase, creating a GSH/superoxide radical sink. In the present studies, an excessive production of superoxide/hydrogen peroxide induced by the hemolytic concentration of MAQ-NOH, in

the presence of kinetically-free iron derived from methemoglobin, could account for the observed hydroxyl radical signal.

It is apparent that while the GSH/superoxide radical sink concept explains satisfactorily the dependence of the hydroxyl radical signal on cellular GSH levels, it does not provide an explanation for the initiation of the radical series or how methemoglobin formation is provoked by MAQ-NOH. The relative stability of MAQ-NOH in buffer ($T_{1/2}$ ca. 4 min) as compared with its instability in the red cells ($T_{1/2} < 2$ min) suggests that a spontaneous reaction with molecular oxygen (i.e., auto-oxidation) to generate the initial radical species is unlikely, and that in some fashion, hemoglobin or other cellular constituents are needed to initiate radical formation. Similarly, comparison of methemoglobin levels in the presence and absence of Na_2S (fig. 4.9) indicates that the comproportionation of ferryl heme is not a major contributor to MAQ-NOH-induced methemoglobin formation, and hence that methemoglobin must arise by a different pathway.

While neither postulate alone provides a satisfactory explanation, a combination appears to be compatible with the current data. Thus, the very rapid disappearance of MAQ-NOH in red cells and rapid formation of methemoglobin (Bolchoz et al., 2001) suggest that the interaction of MAQ-NOH and hemoglobin could generate methemoglobin and a compound-centered free radical, such as a quinoline hydronitroxide radical. The generation of this species may be analogous to the formation of the phenylhydronitroxide radical form of phenylhydroxylamine described by Mason and colleagues (Maples et al., 1990). Once formed, the nitroxide free radical could react with GSH to form a thiyl radical, which in turn, could generate a superoxide radical and lead

to hydrogen peroxide formation via superoxide dismutase (Fridovich, 1975). As noted above, kinetically-free iron derived from methemoglobin could catalyze hydroxyl radical formation.

Although this combined schema permits rationalization of the available data, it should be noted that there are major discrepancies. First, we have been unable to detect a compound-centered radical derived from MAQ-NOH in the red cell incubates. Of interest, aerobic incubation of MAQ-NOH in PBSG buffer alone (without the iron chelator, DTPA) gives rise to a radical species that can be detected by EPR (data not shown). The identity of this species and its relevance to hemotoxicity are currently under investigation. Second, we have been unable to clearly detect a glutathione thiyl radical in MAQ-NOH incubates. This is of concern since previous analogous studies with phenylhyrdroxylamine demonstrated the presence of thiyl radicals (Maples et al., 1990; Bradshaw et al., 1995). However, since the EMPO-hydroxyl and EMPO-GSH thiyl radical adduct signals are similar, with a characteristic 1:2:2:1 signal pattern, simulation experiments are necessary to determine if this signal is present and just masked by the hydroxyl signal.

A third major concern is the lack of correlation between the very rapid disappearance of MAQ-NOH and the apparent steady production of hydroxyl radical for at least 20 min after addition of the MAQ-NOH to the red cells. The fact that hydroxyl radical continues to be generated long after MAQ-NOH has disappeared argues strongly that MAQ-NOH induces the formation of an as yet undetected, more stable intermediate. Support for the existence of such a species can also be seen in MAQ-NOH-treated, GSH-depleted red cells. Although the hydroxyl radical can no longer be detected, addition of the lipid

soluble sulfhydryl-donating compound, cysteamine, leads to the trapping of a cysteamine thiyl radical by EMPO (Bolchoz et al., unpublished observations). Thus even in red cells in which the hydroxyl radical is not detectable, a precursor species more stable that MAQ-NOH must be present.

The role of hydrogen peroxide in the hemolytic process is also unclear. It is well known that hydrogen peroxide reacts with hemoglobin to form ferryl heme and hence that ferryl heme levels in the red cells reflect the generation of hydrogen peroxide in excess of its metabolic clearance (Kanner and Harel, 1985b; Kanner and Harel; 1985a; Harel and Kanner, 1988). Thus the formation of ferrylhemoglobin in these red cells may be taken as evidence for the formation of excess hydrogen peroxide under MAQ-NOH-induced hemolytic conditions. However, it has long been thought that glutathione peroxidase plays a major role in the removal of hydrogen peroxide from the red cell (Jacob, 1965). Thus, in the present studies, depletion of GSH from the red cells should have decreased hydrogen peroxide elimination by glutathione peroxidase, resulting in enhanced hydrogen peroxide levels and enhanced ferryl heme formation. Experimentally, depletion of red cell GSH had no effect on the production of ferryl heme (fig. 4.8A), implying no significant change in hydrogen peroxide levels. Since ferryl heme appears to play little or no role in MAQ-NOH-induced methemoglobin formation (fig. 4.8B), the lack of enhancement in ferryl heme levels is not due to enhanced removal by comproportionation to methemoglobin. The data are consistent with the proposal by Eaton and colleagues (Eaton, 1991) that catalase plays a more significant role in controlling red cell peroxide levels than has previously been considered.

In summary, the present studies clearly demonstrate that under hemolytic conditions, MAQ-NOH generates three active oxygen species in rat red cells: hydroxyl radical, hydrogen peroxide and ferryl heme, each of which has the chemical potential to inflict the initial oxidant injury to the red cell that ultimately leads to its premature sequestration by the spleen. However, enhancement of the susceptibility of the cells by prior depletion of cellular GSH did not increase the levels of any of these species and hence these data alone do not allow us to conclude which, if any, of these oxidants could be causal in MAQ-NOH hemotoxicity. Of particular interest, the marked discrepancy between the rapid disappearance of MAQ-NOH from the red cell incubate and the sustained oxidant stress for at least 20 min suggests the presence of an, as yet, undetected pro-oxidant species derived from MAQ-NOH under hemolytic conditions.

CHAPTER 5

Summary

Summary

Hemolytic anemia has been recognized as a toxic side effect of a wide variety of drugs and environmental chemicals for more than 50 years (Cordes, 1926). While the precise mechanism in which these agents elicit a hemolytic response remains unclear, it is generally accepted that oxidative stress plays a central role. Support for the oxidative stress concept lies in a number of observations made in early mechanistic studies (for review, see Beutler, 1959). These include: 1) loss of cellular reduced glutathione following exposure to these agents, 2) oxidation of oxyhemoglobin to methemoglobin, and 3) cells that are unable to maintain adequate levels of the reductant NADPH (i.e., G6PD deficient cells) when faced with an oxidative challenge are the most sensitive to these agents. In addition, these studies established that metabolites were responsible for the hemolytic activity of the parent compound.

While the crucial role for metabolism in primaquine-induced hemolytic anemia is well accepted, the hemotoxic metabolite(s) have not been identified. Furthermore, the mechanism underlying this toxicity is not fully understood. This is in one part due to multiple oxidation sites on the primaquine molecule that give rise to a number of possible primary metabolites that may be further metabolized. Secondly, many of these putative metabolites are known to be chemically unstable in biological systems (Strother et al., 1981; Idowu et al., 1995). In addition, investigators lack a good primaquine-sensitive experimental animal model (Lee et al., 1981).

It has long been suggested that primaquine hemotoxicity is mediated by redox active phenolic metabolites of the parent compound (Tarlov et al., 1962). These metabolites are believed to redox cycle within the red cell to their respective quinone/ quinoneimine structure resulting in the formation of reactive oxygen species (Link et al., 1985). In support, 5-OH-PQ and 5,6-DHPQ have been detected in the biological fluid of experimental animals administered primaquine (Strother et al., 1981; Strother et al., 1984; Idowu et al., 1995), and these metabolites have been shown to decrease erythrocytic GSH levels and oxidize hemoglobin to methemoglobin in both normal and G6PD-defecient erythrocytes (Fraser and Vesell, 1968; Agarwal et al., 1988; Fletcher et al., 1988). However, these derivatives have not been detected in humans (Baty et al., 1975; Mihaly et al., 1984).

Alternatively, previous studies with hemolytic arylamines such as aniline (Harrison and Jollow, 1986), phenacetin (Jensen and Jollow, 1991), and dapsone (Grossman and Jollow, 1988), have shown that their N-hydroxy metabolites are the hemotoxic species. Evidence suggests that these agents evoke an oxidative stress within the red cell through a cyclic oxidation-reduction reaction that occurs between the arylhydroxylamine, oxyhemoglobin, and molecular oxygen yielding the nitrosoarene, methemoglobin, and partially reduced forms of oxygen, respectively (Rostorfer and Cormier, 1957; Kiese, 1974). In support, extensive studies on the mechanism of DDS-NOH-induced hemolytic anemia revealed that this metabolite induced a variety of oxidative events in rat and human erythrocytes under hemolytic conditions (Grossman and Jollow, 1988; Grossman et al., 1992; McMillan et al., 1995; Bradshaw et al., 1997). These include: 1) rapid and extensive depletion of GSH with a concomitant formation of GSH-protein mixed

disulfides, 2) formation of disulfide-linked hemoglobin adducts to certain membrane skeletal proteins, 3) oxidation of hemoglobin, and 4) generation of hydroxyl radicals. In contrast, DDS-NOH did not oxidatively damage red cell membrane lipids (McMillan et al., 1998). Collectively, these observations led to the concept (fig. 1.5) that arylhydroxylamines redox cycle within the red cell, generating oxygen-centered free radicals, which in turn form GSH and hemoglobin thiyl radicals. The hemoglobin thiyl radicals are believed to further react with the sulfhydryl groups of the membrane skeletal proteins to form hemoglobin-skeletal protein adducts and ultimately mark the red cell for premature removal by the spleen (Grossman et al., 1992).

In light of these studies, this dissertation examines the possible role of an N-hydroxy metabolite in primaquine-induced hemotoxicity. The experimental approach was to determine if this type of metabolite is formed metabolically, to establish its hemolytic activity, to characterize the pattern of oxidative damage induced in the red cell particularly to membrane lipids and cytoskeletal proteins and to identify the nature of the oxidative stress generated by this metabolite in the red cell.

Formation and hemotoxicity of primaquine N-hydroxy metabolite

As 6-methoxy-8-aminoquinoline (6-MAQ) is one of two known human primaquine metabolites, rat and human microsomes were analyzed for their capacity to N-hydroxylate this metabolite. As seen in figure 2.5, incubation of 6-MAQ with rat and human liver microsomes resulted in the formation of a single metabolite that was identified by mass spectrometry to be MAQ-NOH (fig. 2.7). MAQ-NOH was further shown to be hemolytic *in vivo* as measured by the decreased survival of ^{51}Cr-labeled

erythrocytes in rats. Of particular interest, incubation of MAQ-NOH with ^{51}Cr-labeled erythrocytes *in vitro* resulted in a concentration-dependent increase (EC_{50} 350 μM) in their rate of removal when administered to a group of isologous rats. MAQ-NOH also induced a concentration-dependent increase in methemoglobin levels in rat red cells when exposed *in vitro*. Collectively these data suggest that the 6-MAQ can be metabolized to MAQ-NOH and that this metabolite has the requisite properties to play a role in the hemolytic activity of primaquine. However, the contribution of MAQ-NOH to primaquine-induced hemolytic anemia *in vivo* remains to be assessed.

Hemolytic dose-respose relationship

In regard to the *in vitro* exposure/ *in vivo* survival assay employed in this aim, it is important to note that the metabolite concentrations needed to elicit a hemolytic response *in vitro* may not be extrapolated to define the concentration of these drugs needed to elicit a hemolytic response *in vivo*. One reason for this is that the hemolytic activity of these agents is being tested in G6PD-normal rat erythrocytes. Since these red cells have normal GSH levels and can maintain NADPH levels and hence antioxidant enzyme activity when challenged with an oxidative stress, we must use metabolite concentrations that not only deplete GSH, but also generate enough oxidative stress to overwhelm the antioxidant enzymes in order to elicit a toxic response.

Secondly, drug-induced hemolytic anemia is believed to be the result of an accumulation of multiple "hits" after which a threshold is reached the cells are removed from circulation. Thus hemolytic damage would be proportional to the area under the metabolite plasma concentration vs time curve and not the peak metabolite plasma levels,

as has been illustrated for the hemolytic metabolite of dapsone, dapsone hydroxylamine (Grossman and Jollow, 1988). Primaquine has a half-life of about 6 hrs (Mihaly et al., 1984), therefore it is conceivable that 6-MAQ and MAQ-NOH will be produced at a low but steady rate. Since primaquine is usually administered chronically, the area under the MAQ-NOH plasma concentration versus time curve may be significant, particularly in a G6PD-deficient individual. However, since the red cells in our survival assay are only exposed to the metabolites *in vitro* for 2 hrs, higher than physiological relevant concentrations of metabolites must be used.

On the contrary, this assay does provide a method in which shorter incubation times can be used to determine the relative potencies of direct acting hemolytic compounds. In addition, *in vitro* experimental conditions are established that result in the *in vivo* premature sequestration of red cells. Thus parallel studies may be conducted to examine the biochemical changes that occur in the red cells under these conditions and their possible role in marking the red cell for removal ascertained.

Oxidative effects of MAQ-NOH in rat erythrocytes under hemolytic conditions

Being that MAQ-NOH is an arylhydroxylamine like DDS-NOH, it was expected to behave in a similar nature in the red blood cell. However, in marked contrast to the DDS-NOH, MAQ-NOH was shown to induce significant lipid peroxidation in rat erythrocytes with little effect on the membrane skeletal proteins under equally hemolytic conditions. Of particular interest, MAQ-NOH exposure resulted in hemoglobin monomer association with the erythrocyte lipid membrane. The monomer could not be removed despite multiple rigorous washes in phosphate buffer suggesting it was bound either non-

covalently (hydrophobic interactions) or covalently to the lipid bilayer. As this monomer has also been seen in red cells treated with other hemolytic agents, for example DDS-NOH, phenylhydrazine, and divicine, the nature of the monomer association with the red cell membrane and its possible role in marking the cell for splenic sequestration as apposed to or in conjunction with the cytoskeletal adducts or lipid peroxidation becomes of interest.

MAQ-NOH was also shown to differ from DDS-NOH in its effects on GSH in rat red cells. As illustrated in figure 3.1, MAQ-NOH only moderately and transiently decreased GSH levels. In fact the concentration-response curve for MAQ-NOH GSH depletion is far to the right of its hemolytic response curve suggesting that GSH depletion is not a prerequisite for oxidative hemolytic damage.

Oxidative effects of MAQ-NOH in GSH-depleted rat erythrocytes

In an effort to investigate the oxidative effects of MAQ-NOH on red cells that more closely resemble G6PD deficient red cells (i.e. cells with reduced levels of GSH (Beutler, 1978), rat red cells were treated with DEM to deplete GSH prior to exposure to hemolytic concentrations of MAQ-NOH. As seen in figure 3.5, GSH depletion markedly enhanced MAQ-NOH hemolytic activity as expected. However, the increase in toxicity was not accompanied by an increase in lipid peroxidation. Surprisingly, MAQ-NOH induced a concentration-dependent increase in membrane skeletal protein hemoglobin adducts in red cells absent of GSH. Collectively these data suggest that MAQ-NOH may mediate its hemolytic activity through more than one mechanism; one that involves lipid

peroxidation in red cells with normal levels of GSH and protein oxidation in red cells with decreased GSH levels, such as those seen in G6PD-deficient individuals.

DEM-treated erythrocytes as a model of G6PD deficiency

Although DEM-treated red cells are more sensitive to oxidative injury, there are some differences between these cells and G6PD-deficient cells. First, GSH levels are known to be decreased in G6PD-deficient cells (Beutler, 1978), however in red cells exposed to DEM, GSH is essentially absent. Though DEM-treated red cells may represent G6PD cells following an oxidative stress. Secondly, DEM does not specifically react with GSH. Sulfhydryl groups on cellular proteins may also be blocked by DEM. In an effort to minimize this possibility, red cell GSH is titrated out with small aliquots of DEM. Furthermore, should any DEM side reactions be occurring in our experiments, this does not appear to effect the *in vivo* survival, levels of lipid peroxidation, or protein electrophoretic pattern of these red cells as indicated in control experiments.

It is also important to note that a more specific method than DEM treatment does exits in which to model G6PD deficiency. This approach involves the use of steroids such as epiandrosterone to non-competitively inhibit G6PD (Marks and Banks, 1960). Our laboratory has previously employed this technique to demonstrate an enhanced sensitivity of G6PD inhibited rat red cells versus uninhibited cells to DDS-NOH (Grossman et al., 1995). The difference in sensitivity in these experiments was comparable to that seen by Degowin et al. in G6PD-deficient and normal individuals given dapsone (Degowin et al., 1966). However, despite multiple attempts, we have been unable to successfully inhibit G6PD to the extent necessary to represent an A- G6PD deficient individual (>95%

inhibition) since these studies. Efforts to elucidate the reason for this lack of response are being continued.

Effect of MAQ-NOH on free radical formation in rat erythrocytes

In light of the critical role of oxidative stress in drug-induced hemolytic anemia, additional experiments were conducted to detect and identify any free radicals generated in rat red cells exposed to hemolytic concentrations of MAQ-NOH. EPR analysis using the spin trap 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) revealed that in normal rat red cells, MAQ-NOH generated a hydroxyl radical adduct signal in a concentration-dependent manner. Surprisingly, this radical was continuously generated for up to 20 min despite that MAQ-NOH has a half-life of less than a minute in blood (Bolchoz et. al., unpublished data). Furthermore, in conjunction with the above studies, red cells treated with DEM to deplete GSH prior to MAQ-NOH exposure were also examined by EPR. Interestingly, no radical adduct signal was detected under these conditions in rat red cells suggesting that MAQ-NOH-induced hydroxyl radical formation was GSH dependent. Of particular importance, these data raise the possibility that a third type of radical (i.e. compound-centered radical) may be initially generated in response to MAQ-NOH that reacts with GSH to ultimately give rise to a hydroxyl radical. This radical may also be more cytotoxic than the hydroxyl radical as MAQ-NOH hemolytic activity is known to be markedly enhanced in GSH-depleted red cells. Additional spectrophotometric analysis of MAQ-NOH treated red cells revealed the presence of ferrylhemoglobin, thereby confirming the presence of hydrogen peroxide and by inference superoxide in these cells. The role of ferryl heme species in MAQ-NOH

hemolytic activity remains to be determined. In addition, whether a compound-centered radical is produced in red cells in response to MAQ-NOH and its role in causing the intracellular lesions described above certainly warrants further investigation.

Conclusion

In trying to answer the question of which primaquine metabolite mediates its hemotoxic side effects, the first phase of this project was to determine if the known human primaquine metabolite 6-MAQ could be N-hydroxylated to MAQ-NOH. Evidence is presented to show that MAQ-NOH is not only formed from 6-MAQ, but that it is directly hemotoxic. MAQ-NOH was further demonstrated to be hemolytic *in vivo*. Despite that this metabolite has the requisite properties to mediate parent compound hemolytic anemia, its relative contribution remains unclear. This is largely due to the possible contribution of the redox active phenolic metabolites to the toxicity. In this regard, preliminary studies in our laboratory have demonstrated that the phenolic metabolites of primaquine are also directly hemolytic (fig 5.1). In fact these metabolites appear to be markedly more potent than MAQ-NOH in normal rat erythrocytes. However, there exists no evidence to support that these metabolites are formed in humans. Furthermore, should these metabolites be produced *in vivo*, the possibility exists that they may never reach the circulation to mediate this toxicity due to their extreme chemical instability or further metabolism. In support, 2-aminophenol and 4-aminophenol, the phenolic metabolites of aniline, although directly hemotoxic, were found to be rapidly eliminated by hepatic conjugation and not to contribute to the parent compound hemotoxicity (Harrison and Jollow, 1986; Harrison and Jollow, 1987). Studies

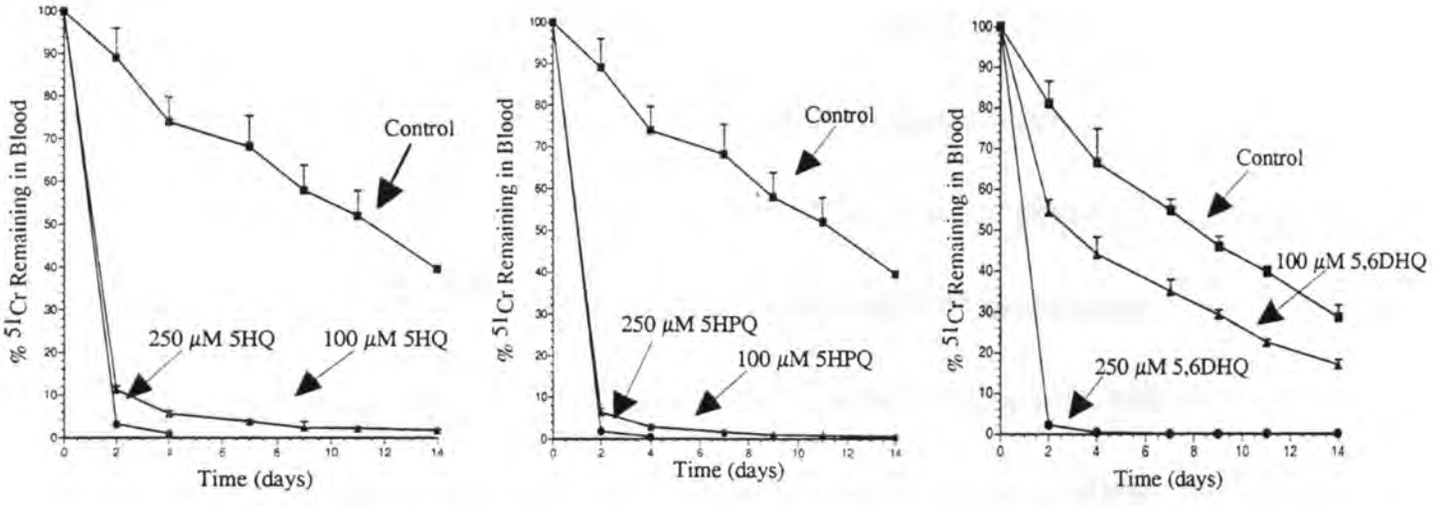

Fig. 5.1. Survival of rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to the phenolic metabolites of primaquine 5HQ, 5-hydroxy-6-methoxy-8-aminoquinoline, 5HPQ, 5-hydroxyprimaquine, and 5,6DHPQ, 5,6-dihydroxyprimaquine. The red cells were incubated with the phenolic metabolites for 2 hr at 37°C; control cells were incubated with vehicle (PBSG) alone. The erythrocytes were then washed and administered i.v. to isologous rats. To blood samples were taken 30 min after administration of the labeled cells. Data points are means ± SD (n=4).

on the metabolic formation and elimination of the phenolic metabolites and on their hemolytic activity *in vivo* are certainly warranted.

Alternately, the phenolic metabolites may play a role in the antimalarial activity of primaquine since oxidative injury to the malaria parasite has been implicated as a possible mode of action. A large concern in the development of more effective tissue schizontocides has been that the antimalarial activity and the hemolytic activity of primaquine may both be mediated by an oxidative stress. However, should these two oxidative stresses be due to two separate types of redox active metabolites, one may speculate that the parent compound could be modified to prevent formation of the hemotoxic species. For example, the phenolic metabolites of primaquine could be formed in the liver where they redox cycle creating an oxidative stress that lethally damages the parasite, and then are quickly eliminated before reaching the circulation. However, the N-hydroxy metabolite may be absorbed into the circulation, where it reacts with hemoglobin creating an oxidative stress that critically damages the red cell.

The second phase of this project focuses on the mechanism of MAQ-NOH-induced hemolytic anemia particularly in comparison to other hemolytic arylhydroxylamines. In these studies MAQ-NOH was shown to induce lipid peroxidation and hemoglobin monomer association with the plasma membrane under hemolytic conditions with little effect on the membrane cytoskeletal proteins. This pattern of oxidative injury is in marked contrast to that seen previously with hemolytic agents such as DDS-NOH (Grossman et al., 1992), phenylhydrazine (Jandl et al., 1960), and divicine (McMillan et al., 2001) particularly with respect to the lack of hemoglobin adducts to the membrane cytoskeletal proteins. Of importance, these data question the relevance of hemoglobin

adducts to membrane cytoskeletal proteins like band 3 in marking the red cell for removal by macrophages of the spleen. Furthermore, while peroxidation of the membrane lipids could certainly mediate macrophage recognition of MAQ-NOH-damaged red cells, it is also possible that the hemoglobin monomer may play a role. Since hemoglobin monomer association with the plasma membrane is a common characteristic for all these hemolytic agents, one may speculate that its association may alter the lipid membrane in a manner that leads to a conformational change in band 3 to trigger macrophage ingestion of the red cell.

In contrast to GSH-normal red cells, MAQ-NOH hemolytic activity was markedly enhanced red cells previously depleted of GSH. The exacerbation of toxicity was accompanied by the formation of hemoglobin adducts to the membrane skeletal proteins with little effect on lipid peroxidation suggesting that both of these mechanisms can signal macrophage recognition of damaged red cells. Moreover, should MAQ-NOH be the metabolite responsible for primaquine-induced hemolytic anemia, these data would provide for the first time a biochemical basis to explain the difference in sensitivity of G6PD-deficient individuals and G6PD-normal individuals to primaquine and dapsone (Degowin et al., 1966). It is hoped that exploitation of this unique hemolytic agent in future studies will ultimately provide some additional insight as to mechanism of drug-induced hemolytic anemia.

It is of particular interest that despite the differences in the pattern of hemolytic injury induced by DDS-NOH and MAQ-NOH, both of these agents have been shown to generate a hydroxyl radical under hemolytic conditions (Bradshaw et al., 1997). However, in the case of MAQ-NOH, the hydroxyl radical generation is GSH-dependent.

Thus despite the chemical similarities of these two compounds, they appear to react differently within the red cell. One possibility is that MAQ-NOH exposure results in the generation of a compound-centered radical that is scavenged by GSH and transferred to oxygen. This idea is supported by the enhanced hemolytic activity of MAQ-NOH in the absence of GSH and a hydroxyl radical.

In a continued effort to elucidate the mechanism of MAQ-NOH-induced hemolytic activity our lab has recently discovered that incubation of rat erythrocytes with the exogenous aminothiol cysteamine, while exposed to MAQ-NOH, does not protect the red cells from MAQ-NOH-induced hemolytic injury as seen previously with DDS-NOH (fig. 5.2). To the contrary, 5 mM cysteamine significantly enhanced MAQ-NOH hemolytic activity when co-incubated in rat erythrocytes. This was rather unexpected as cysteamine, like GSH, is known to directly scavenge reactive free radicals and to maintain cellular sulfhydryl groups in a reduced form thereby protecting the red cells from oxidative damage. Additional experiments in which cysteamine was incubated with MAQ-NOH in rat red cells previously depleted of GSH revealed that cysteamine also markedly exacerbated MAQ-NOH hemolytic damage in GSH-depleted cells (fig. 5.3). In light of these findings, our lab intends to examine the effects of cysteamine on MAQ-NOH-induced alterations of the membrane lipids and proteins in both normal and GSH-depleted rat red cells. We also propose to use EPR to examine the free radicals produced in rat red cells under these various conditions as well as to continue our attempts to trap any compound-centered radicals in MAQ-NOH-treated erythrocytes.

In summary, while it is difficult to speculate on the molecular mechanism of MAQ-NOH-induced hemolytic activity at this point, these data clearly demonstrate that the

mechanism differs from that of DDS-NOH, despite their similar chemical structure. It is hoped that exploitation of this unique hemolytic agent in future studies will ultimately provide some additional insight as to mechanism of drug-induced hemolytic anemia.

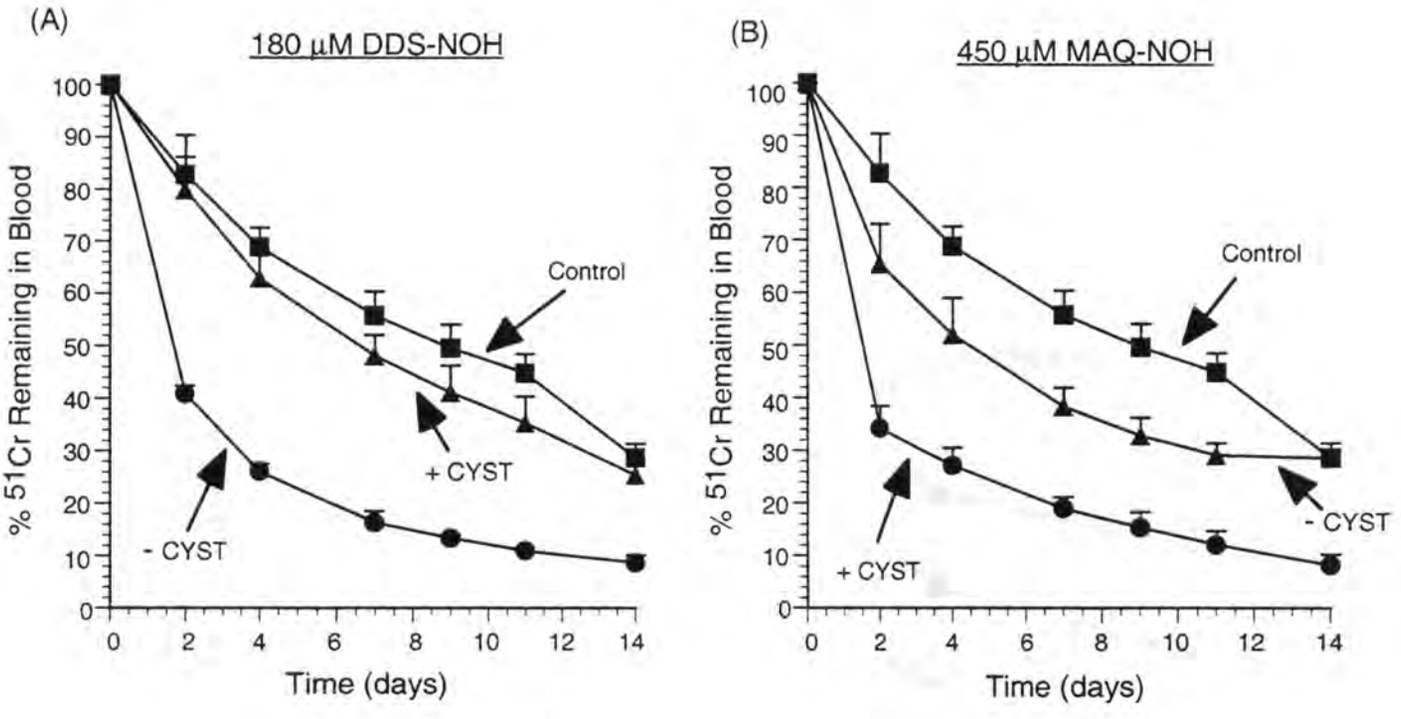

Fig. 5.2. The effect of cysteamine on the survival of rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to (A) DDS-NOH (180 μM) or (B) MAQ-NOH (450 μM). The red cells were incubated with 5 mM cysteamine and either DDS-NOH or MAQ-NOH for 2 hr at 37°C; control cells were incubated with vehicle (PBSG) alone. The erythrocytes were then washed and administered i.v. to isologous rats. To blood samples were taken 30 min after administration of the labeled cells. Data points are means $\pm$ SD (n=4).

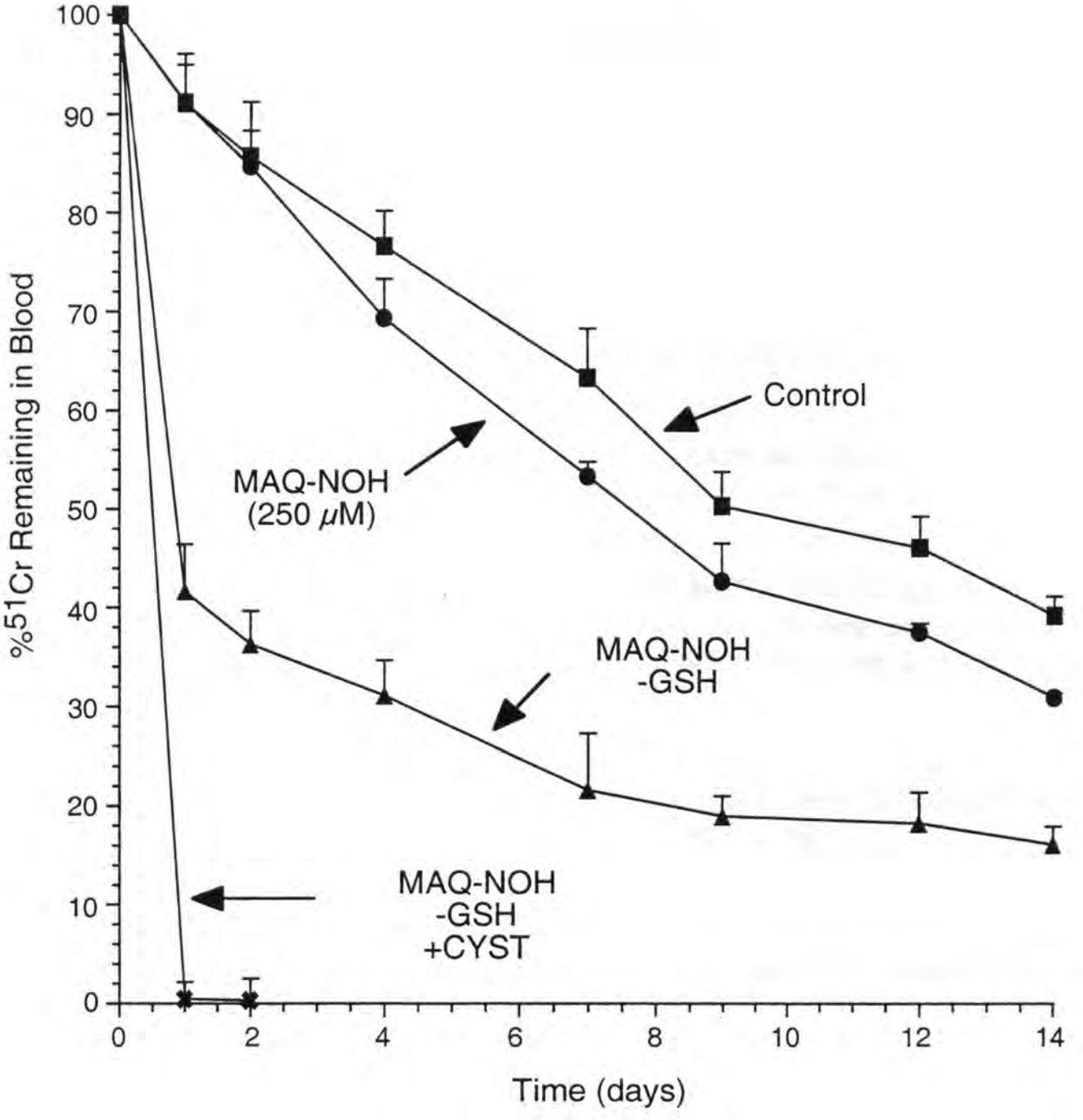

Fig. 5.3. The effect of cysteamine on the survival of GSH-depleted ^{51}Cr-labeled rat erythrocytes *in vivo* after *in vitro* exposure to MAQ-NOH (250 μM). Rat red cells were depleted of GSH (>90%) using DEM and incubated with either MAQ-NOH alone or 5 mM cysteamine and MAQ-NOH for 2 hr at 37°C; control cells were incubated with vehicle (20 μl DMSO) alone. The erythrocytes were then washed and administered i.v. to isologous rats. To blood samples were taken 30 min after administration of the labeled cells. Data points are means ± SD (n=4).

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